THE FUNCTION AND MECHANISMS OF ACTION OF GHRELIN AND OBESTATIN IN OVARIAN CANCER

Carina Maree Walpole

Bachelor of Applied Science (Hons)

Bachelor of Biotechnology Innovations (Hons)

Institute of Health and Biomedical Innovation

Faculty of Health

Queensland University of Technology

A thesis submitted for the degree of Doctor of Philosophy of the Queensland University of Technology

2012

KEYWORDS

Ghrelin, obestatin, GHSR1a, GHSR1b, migration, invasion, proliferation, attachment, 2D-DIGE, real time RT-PCR, PCR, signalling, pathways, MAPK, ERK1/2, ovarian cancer.

ABSTRACT

In this study, we have demonstrated that the preproghrelin derived hormones, ghrelin and obestatin, may play a role in ovarian cancer. Ghrelin and obestatin stimulated an increase in cell migration in ovarian cancer cell lines and may play a role in cancer progression. Ovarian cancer is the leading cause of death among gynaecological cancers and is the sixth most common cause of cancer-related deaths in women in developed countries. As ovarian cancer is difficult to diagnose at a low tumour grade, two thirds of ovarian cancers are not diagnosed until the late stages of cancer development resulting in a poor prognosis for the patient. As a result, current treatment methods are limited and not ideal. There is an urgent need for improved diagnostic markers, as well better therapeutic approaches and adjunctive therapies for this disease.

Ghrelin has a number of important physiological effects, including roles in appetite regulation and the stimulation of growth hormone release. It is also involved in regulating the immune, cardiovascular and reproductive systems and regulates sleep, memory and anxiety, and energy metabolism. Over the last decade, the ghrelin axis, (which includes the hormones ghrelin and obestatin and their receptors), has been implicated in the pathogenesis of many human diseases and it may t may also play an important role in the development of cancer. Ghrelin is a 28 amino acid peptide hormone that exists in two forms. Acyl ghrelin (usually referred to as ghrelin), has a unique *n*-octanoic acid post-translational modification (which is catalysed by ghrelin *O*-acyltransferase, GOAT), and desacyl ghrelin, which is a non-octanoylated form. Octanoylated ghrelin acts through the growth hormone secretagogue receptor type 1a (GHSR1a). GHSR1b, an alternatively spliced isoform of GHSR, is C-terminally truncated and does not bind ghrelin. Ghrelin has been implicated in the pathophysiology of a number of diseases Obestatin is a 23 amino acid, C-terminally amidated peptide which is derived from preproghrelin. Although GPR39 was originally thought to be the obestatin receptor this has been disproven, and its receptor remains unknown. Obestatin may have as diverse range of roles as ghrelin. Obestatin improves memory, inhibits thirst and anxiety, increases pancreatic juice secretion and has cardioprotective effects. Obestatin also has been shown to regulate cell proliferation, differentiation and apoptosis in some cell types.

Prior to this study, little was known regarding the functions and mechanisms of action ghrelin and obestatin in ovarian cancer. In this study it was demonstrated that the full length ghrelin, GHSR1b and GOAT mRNA transcripts were expressed in all of the ovarian-derived cell lines examined (SKOV3, OV-MZ-6 and hOSE 17.1), however, these cell lines did not express GHSR1a. Ovarian cancer tissue of varying stages and normal ovarian tissue expressed the coding region for ghrelin, obestatin, and GOAT, but not GHSR1a, or GHSR1b. No correlations between cancer grade and the level of expression of these transcripts were observed.

This study demonstrated for the first time that both ghrelin and obestatin increase cell migration in ovarian cancer cell lines. Treatment with ghrelin (for 72 hours) significantly increased cell migration in the SKOV3 and OV-MZ-6 ovarian cancer cell lines. Ghrelin (100 nM) stimulated cell migration in the SKOV3 (2.64 +/- 1.08 fold, $p < 0.05$) and OV-MZ-6 (1.65 +/- 0.31 fold, $p < 0.05$) ovarian cancer cell lines, but not in the representative normal cell line hOSE 17.1. This increase in migration was not accompanied by an increase in cell invasion through Matrigel. In contrast to other cancer types, ghrelin had no effect on proliferation. Ghrelin treatment (10nM) significantly decreased attachment of the SKOV3 ovarian cancer cell line to collagen IV (24.7 +/- 10.0 %, $p < 0.05$), however, there were no changes in attachment to the other extracellular matrix molecules (ECM) tested (fibronectin, vitronectin and collagen I), and there were no changes in attachment to any of the ECM molecules in the OV-MZ-6 or hOSE 17.1 cell lines. It is, therefore, unclear if ghrelin plays a role in cell attachment in ovarian cancer.

As ghrelin has previously been demonstrated to signal through the ERK1/2 pathway in cancer, we investigated ERK1/2 signalling in ovarian cancer cell lines. In the SKOV3 ovarian cancer cell line, a reduction in ERK1/2 phosphorylation (0.58 fold +/- 0.23, $p < 0.05$) in response to 100 nM ghrelin treatment was observed, while no significant change in ERK1/2 signalling was seen in the OV-MZ-6 cell line with treatment. This suggests that this pathway is unlikely to be involved in mediating the increased migration seen in the ovarian cancer cell lines with ghrelin treatment.

In this study ovarian cancer tissue of varying stages and normal ovarian tissue expressed the coding region for obestatin, however, no correlation between cancer

grade and level of obestatin transcript expression was observed. In the ovarian-derived cell lines studied (SKOV3, OV-MZ-6 and hOSE 17.1) it was demonstrated that the full length preproghrelin mRNA transcripts were expressed in all cell lines, suggesting they have the ability to produce mature obestatin.

This is the first study to demonstrate that obestatin stimulates cell migration and cell invasion. Obestatin induced a significant increase in migration in the SKOV3 ovarian cancer cell line with 10 nM (2.80 +/- 0.52 fold, $p < 0.05$) and 100 nM treatments (3.12 +/- 0.68 fold, $p < 0.05$) and in the OV-MZ-6 cancer cell line with 10 nM (2.04 +/- 0.10 fold, $p < 0.01$) and 100 nM treatments (2.00 +/- 0.37 fold, $p < 0.05$). Obestatin treatment did no affect cell migration in the hOSE 17.1normal ovarian epithelial cell line. Obestatin treatment (100 nM) also stimulated a significant increase in cell invasion in the OV-MZ-6 ovarian cancer cell line (1.45 fold +/- 0.13, $p < 0.05$) and in the hOSE17.1 normal ovarian cell line cells (1.40 fold +/- 0.04 and 1.55 fold +/- 0.05 respectively, $p < 0.01$) with 10 nM and 100 nM treatments. Obestatin treatment did not stimulate cell invasion in the SKOV3 ovarian cancer cell line. This lack of obestatin-stimulated invasion in the SKOV3 cell line may be a cell line specific result.

In this study, obestatin did not stimulate cell proliferation in the ovarian cell lines and it has previously been shown to have no effect on cell proliferation in the BON-1 pancreatic neuroendocrine and GC rat somatotroph tumour cell lines. In contrast, obestatin has been shown to affect cell proliferation in gastric and thyroid cancer cell lines, and in some normal cell lines. Obestatin also had no effect on attachment of any of the cell lines to any of the ECM components tested (fibronectin, vitronectin, collagen I and collagen IV).

The mechanism of action of obestatin was investigated further using a two dimensional-difference in gel electrophoresis (2D-DIGE) proteomic approach. After treatment with obestating (0, 10 and 100 nM), SKOV3 ovarian cancer and hOSE 17.1 normal ovarian cell lines were collected and 2D-DIGE analysis and mass spectrometry were performed to identify proteins that were differentially expressed in response to treatment. Twenty-six differentially expressed proteins were identified and analysed using Ingenuity Pathway Analysis (IPA). This linked 16 of these

proteins in a network. The analysis suggested that the ERK1/2 MAPK pathway was a major mediator of obestatin action. ERK1/2 has previously been shown to be associated with obestatin-stimulated cell proliferation and with the anti-apoptotic effects of obestatin.

Activation of the ERK1/2 signalling pathway by obestatin was, therefore, investigated in the SKOV3 and OV-MZ-6 ovarian cancer cell lines using anti-active antibodies and Western immunoblots. Obestatin treatment significantly decreased ERK1/2 phosphorylation at higher obestatin concentrations in both the SKOV3 (100 nM and 1000 nM) and OV-MZ-6 (1000 nM) cell lines compared to the untreated controls. Currently, very little is known about obestatin signalling in cancer.

This thesis has demonstrated for the first time that the ghrelin axis may play a role in ovarian cancer migration. Ghrelin and obestatin increased cell migration in ovarian cancer cell lines, indicating that they may be a useful target for therapies that reduce ovarian cancer progression. Further studies investigating the role of the ghrelin axis using *in vivo* ovarian cancer metastasis models are warranted.

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
2D-DIGE	Two Dimensional-Difference in Gel Electrophoresis
µg	Microgram(s)
µl	Microlitre(s)
µM	Micromolar
AA	Amino Acid
ACC	Acetyl-CoA Carboxylase
ACN	Acetonitrile
ACTH	Adrenocorticotrophic Hormone
ADH	Antidiuretic Hormone
AgRP	Agouti Related Peptide
AMPK	AMP-Activated Protein Kinase
ANOVA	Analysis Of Variance
BAEC	Bovine Aortic Endothelial Cells
Bax	Bcl-2-Associated X Protein
BCA	Bicinchoninic Acid
Bcl-2	B-Cell Lymphoma 2
BMD	Bone Mineral Density
BMI	Body Mass Index
bp	Base pair(s)
BSA	Bovine Serum Albumin
BVA	Biological Variation Analysis
CA125	Cancer Antigen 125
Ca^{2+}	Calcium
CAD	Collision-Activated Dissociation
CaMKII	Calcium/Calmodulin-Dependent Kinase II
CaMKK2	Calmodulin Kinase Kinase 2
cAMP	Cyclic Adenosine Monophosphate
CART	Cocaine and Amphetamine Related Transcript
CB1	Cannabinoid Receptor Type 1
cDNA	Complementary DNA

cGMP	Cyclic Guanosine Monophosphate
CHAPS	3-[(3-Cholamidopropyl)Dimethylammonio]-2-Hydroxy-1-Propanesulfonate
CI	Confidence Interval
CL	Corpus Luteum
cm	centimetre
CMEC	Cardiac Microvascular Endothelial Cell
CPT1	Carnitine Palmitoyl Transferase 1
DIA	Differential-In-gel Analysis
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor
eNOS	Endothelial Nitric Oxide Synthase
ER	Endoplasmic Reticulum
ERK1/2	Extracellular Signal-Regulated Kinase 1/2
ERK5	Extracellular Signal-Regulated Kinase 5
FCS	Foetal Calf Serum
Fig	Figure
FSH	Follicle Stimulating Hormone
g	Gram(s)
g	G-force
GH	Growth Hormone
GHRL	Ghrelin
GHS	Growth Hormone Secretagogue
GHSR	Growth Hormone Secretagogue Receptor
GHSR1a	Growth Hormone Secretagogue Receptor 1a
GHSR1b	Growth Hormone Secretagogue Receptor 1b
GLP-1R	Glucagon-Like Peptide 1 Receptor
GnRH	Gonadotropin-Releasing Hormone

GOAT	Ghrelin *O*-Acyl Transferase
GPCR	G Protein Coupled Receptor
GPR39	G Protein Coupled Receptor 39
GRB2	Growth Factor Receptor-Bound Protein 2
GSK-3β	Glycogen Synthase Kinase-3Beta
h	Hour(s)
HCl	Hydrogen Chloride
HMVEC	Human Microvascular Endothelial Cell
HPLC	High-Performance Liquid Chromatography
HSP	Heat Shock Protein
HUVEC	Human Umbilical Vein Endothelial Cell
IPA	Ingenuity Pathway Analysis
IPG	Immobilised pH Gradient
IRS-1	Insulin Receptor Substrate 1
kb	Kilo base pair(s)
kDa	Kilo Dalton(s)
LC-MS/MS	Liquid Chromatography-Mass Spectrometry/ Mass Spectrometry
LH	Luteinizing Hormone
LPS	Lipopolysaccharide
M	Molar
M	Molecular Weight
MAPK	Mitogen Activated Protein Kinases
MBOAT	Membrane-Bound *O*-Acyl Transferase
mg/mL	Milligram Per Milliliter
min	Minute(s)
mL	Millilitre(s)
mM	Millimolar
MMP2	Matrix Metalloproteinase-2
mRNA	Messenger Ribonucleic Acid
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear Factor-Kappa B
ng	Nanogram(s)
nm	Nanometres

nM	Nanomolar(s)
NO	Nitric Oxide
NSCLC	Non- Small Cell Lung Carcinoma
NYP	Neuropeptide Y
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PC	Prohormone Convertases
PC1/3	Prohormone Convertases 1/3
PC2	Proprotein Convertases 2
PCNA	Proliferating Cell Nuclear Antigen
PCOS	Polycystic Ovary Syndrome
PCR	Polymerase Chain Reaction
PI3K	Phosphatidylinositol-3-Kinase
PI-PLC	Phosphatidylinositol-Specific Phospholipase C
PKA	Protein Kinase A
PKC	Protein Kinase C
pmoles	Picomoles
POMC	Pro-Opiomelanocortin
PRL	Prolactin
PWS	Prader-Willi Syndrome
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
s	Second(s)
SCLC	Small Cell Lung Carcinoma
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
siRNA	Small Interfering RNA
SNP	Single-Nucleotide Polymorphism
TBS	Tris Buffered Saline
TBS-Tween	Tris Buffered Saline with Tween
TK	Tyrosine Kinase
TMD	Transmembrane Domain

TNF-α	Tumour Necrosis Factor-Alpha
Tris	Tris(hydroxymethyl)aminomethane
U	Unit(s)
UCP2	Uncoupling Protein 2
UFLC	Ultra Fast Liquid Chromatograph
V	Volt(s)
VCAM-1	Vascular Cell Adhesion Molecule 1
VEGF	Vascular Endothelial Growth Factor
v/v	Volume Per Volume
W/gel	Watts Per Gel
w/v	Weight Per Volume
Zn^{2+}	Zinc

LIST OF PUBLICATIONS

Publications related to the work in this thesis

Lisa Chopin, Inge Seim, **Carina Walpole**, and Adrian Herington (2012) The Ghrelin Axis—Does It Have an Appetite for Cancer Progression? *Endocrine Reviews*, 33(6):849-891

Lisa Chopin, **Carina Walpole**, Inge Seim, Peter Cunningham, Rachael Murray, Eliza Whiteside, Peter Josh, and Adrian Herington (2011) Ghrelin and cancer. *Molecular and Cellular Endocrinology* **340**, 65-69.

Inge Seim, **Carina Walpole,** Laura Amorim, Peter Josh, Adrian Herington and Lisa Chopin (2011) The expanding roles of the ghrelin-gene derived peptide obestatin in health and disease. *Molecular and Cellular Endocrinology* **340**, 111–117.

Inge Seim, Laura Amorim, **Carina Walpole,** Shea Carter, Lisa Chopin, and Adrian Herington (2010) Ghrelin gene-related peptides : multifunctional endocrine/autocrine modulators in health and disease. *Clinical and Experimental Pharmacology and Physiology* **37**, 125-131.

Other publications

Ying Dong, Olivia Tan, Daniela Loessner, Carson Stephens, **Carina Walpole,** Glen Boyle, et al. (2010) Kallikrein-related peptidase 7 promotes multicellular aggregation via the alpha 5 beta1 integrin pathway and paclitaxel chemoresistance in serous epithelial ovarian carcinoma. *Cancer Research*, *70*(7), pp. 2624-2636.

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LIST OF CONFERENCE ABSTRACTS AND PRESENTATIONS

Poster presentations

C. Walpole, I. Seim, P. Josh, A. Herington, L. Chopin (2010). Obestatin Promotes Cell Migration in Ovarian Cancer – A Combined *In Vitro* and Proteomic Approach. *Human Proteome World Congress, Sydney, Australia, 19th-24th September.*

C. Walpole, P. Josh, R. Murray, A. Herington, L. Chopin (2009). Obestatin and Ghrelin Function in Ovarian Cancer Cells. *The Third Annual IHBI inspires Postgraduate Student Conference, Gold Coast, Australia, 17th-18th November.*

C. Walpole, P. Josh, R. Murray, A. Herington, L. Chopin (2009). Obestatin and Ghrelin Function in Ovarian Cancer Cells. *The Australian Society for Medical Research postgraduate conference, Brisbane, Australia, 26th May.*

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C. Walpole, R. Murray, A. Herington, L. Chopin (2008) Obestatin and Ghrelin Function in Ovarian Cancer Cell Lines. *The Forth International Congress of the GRS and the IGF Society, Genoa, Italy, 16th-20th September.*

C. Walpole, R. Murray, I. Bennett, D. Papadimos, A. Yeh, P. Jeffery, A. Herington, L. Chopin (2006). The Emerging Role of Ghrelin and the Ghrelin/GHSR axis in Breast Cancer Development. *Third PacRim Breast & Prostate Cancer Meeting, Fraser Island, Australia, 31st October-4th November.*

STATEMENT OF ORIGINAL AUTHORSHIP

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by any other person except where due reference is made.

Signed: QUT Verified Signature

Carina Walpole

Date: 30/08/13

AKNOWLEDGEMENTS

I would firstly like to thank my principal supervisor A/Prof. Lisa Chopin for the opportunity to undertake this PhD. I would also like to acknowledge my principal supervisor A/Prof. Lisa Chopin and my associate Prof. Adrian Herington for their help, support, patience and advice throughout this PhD and for their assistance in writing this thesis.

I would also like to acknowledge the scholarship and travel grant I received from the Queensland University of Technology and the School of Life Sciences; and the Cancer Council Queensland for funding our research group during the course of this study.

In particular I would like to thank Dr Inge Seim for his collaborative work on the real-time RT-PCR and Peter Josh for teaching me 2D-DIGE as well as both for their help with the Ingenuity Pathway analysis. Thank you also to them and the rest of the past and present ghrelin group members Dr Peter Cunningham, Dr Shea Carter, Eddie Parker, Katie Buzacott, Samia Quaif, Dan Abrahmsen, Joey Tsai, Rachael Wight and Laura Amorim, as well as Dr Eliza Whiteside, Patrick Thomas and Grace Eng for their useful scientific input, assistance, rants and for all of their help de-stressing and keeping me sane. I'd also like to acknowledge the invaluable assistance of technical staff Fardad (Dod) Roshanbin, David Smith and Scott Tucker as well as past and present Laboratory Heads Sonya Winnington-Martin and Lorelle Allen that makes doing laboratory work possible.

Finally, thank you to my parents, Mark and Jenny Walpole, and family for their unconditional, love, assistance and support. Thank you very much to my friends, Crystal McGirr, David Pickwell, Janelle Harris, Jon Harris and Ryan Galea for all your support and much need escapism over the years. Last but not least, a special thank you to my boy, Nick Farlow, for all your love, support, understanding, patience and being there for me.

CHAPTER 1

General Introduction

1.1 Introduction

A little more than a decade ago, the growth hormone secretagogue, ghrelin, was successfully isolated by Kojima *et al.* from stomach tissue as the endogenous ligand for the growth hormone secretagogue receptor (GHSR) [1]. Mature ghrelin is a 28 amino acid peptide which may circulate as desacyl ghrelin (unacylated ghrelin), or as the octanoylated form known as acyl ghrelin (or ghrelin) [1]. The acylation of ghrelin is a unique post-translational modification mediated by the enzyme ghrelin *O*-acyltransferase (GOAT) [2, 3]. Since the original discovery of ghrelin as a growth hormone (GH) releasing hormone, it has been established that it is ubiquitously expressed in the body and has a wide array of functions [4], including a role in appetite regulation [5]. In addition to being an orexigenic signal, ghrelin is involved in regulating the immune, cardiovascular and reproductive systems and regulates sleep, memory and anxiety, energy metabolism and bone, and plays a role in cell proliferation, apoptosis, migration and angiogenesis [4]. Several years after the discovery of ghrelin, Zhang *et al.* identified obestatin as a second peptide hormone which is derived from the preproghrelin peptide and encoded by the ghrelin gene (*GHRL*) [6]. Although obestatin was originally thought to oppose the effects of ghrelin on feeding, this finding proved difficult to replicate and has become controversial [7]. Despite this, obestatin is likely to be as widely expressed as ghrelin and may have a diverse range of functions. Obestatin has also been shown to have roles in thirst, adiposity, pancreatic function, reproduction, the cardiovascular system, sleep, memory, anxiety, and cell proliferation and apoptosis [7]. Some of these functions may be complementary, and a number have been shown to be similar to those of ghrelin including its role in sleep [8], memory [9] and cell proliferation [10-14].

There is increasing evidence that the ghrelin axis plays a role in the progression and development of cancer [15]. Components of the ghrelin axis are expressed locally in a range of different cancers including astrocytoma [16], breast [17, 18], colorectal [19, 20], gastric[11, 19, 21-24], liver [20, 25], lung [26-29], pancreatic [30, 31], pituitary [32-37], prostate [38-40], renal [41], testicular [42], thyroid [43, 44] and ovarian cancer [45, 46] (Tables 1.3 and 1.4). There is emerging evidence that the ghrelin axis may alter functions which are critical to cancer progression, including cell proliferation [11, 17, 25, 38-40, 47-51], apoptosis [47, 49, 52, 53], and cell

migration [48, 54, 55] and invasion [48, 54]. In cancer cell lines, ghrelin has been demonstrated to increase [17, 25, 38-40, 47-51, 54, 56, 57], or decrease [38, 44, 53, 56] cell proliferation, to have anti-apoptotic effects [47, 49, 52], and to increase cell migration [48, 54, 55] and invasion [48, 54]. Although there have been few studies into the role of obestatin in cancer, it has effects on cell proliferation [11, 56]. The ghrelin axis, therefore, may play a role in promoting progression of a number of cancers [15]. There have been few studies regarding the ghrelin axis in ovarian cancer and we hypothesise that ghrelin and obestatin may stimulate processes related to cancer progression in this disease.

1.2 Ovarian cancer

Ovarian cancer is the leading cause of death among gynaecological cancers and is the sixth most common cause of cancer-related deaths after breast, lung, colon, pancreatic and stomach cancer in women in developed countries [58]. In Australia, it is the 9th most commonly diagnosed cancer in women, and it is the second most commonly diagnosed gynaecological cancer [59]. Ovarian cancer is the 4th leading cause of cancer disease burden for females in Australia accounting for 12,900 disability-adjusted life years in 2010 [59]. It has an incidence of 9.4 per 100 000 [58], and the risk of ovarian cancer increases with age. The incidence increases dramatically after 50 years of age, when 81% of all new cases of ovarian cancer are diagnosed [59]. There has been less than a 1% decline in the incidence of ovarian cancer over the past 2 decades and the mortality rate has remained largely unchanged [60].

There are three major types of ovarian cancer, defined by their tissue of origin. Of all cases of ovarian cancer diagnosed, 85-95% are an epithelial histotype, 5-8% are stromal and 3-5% are germ cell in origin [61, 62]. There are a number of different subtypes of each of these types of ovarian cancer. There are three major invasive types of epithelial cancers, which are defined as serous (75%-80%), mucinous (10%) and endometrioid (10%) ovarian cancer [61]. Ovarian cancer, like other cancers, is graded according to tumour development, as stages I-IV, with stage IV representing the most advanced disease (Table 1.1). The stage of the cancer at diagnosis significantly affects the five year survival rate of the patient and influences the choice of treatment [62-64].

Table 1.1 International Federation of Gynaecology and Obstetrics Stages of ovarian cancer showing the spread of cancer according to the stage and the five year prognosis. Adapted from Roett *et.al.* 2009 [65]

Stage	Spread	5-year survival rate
I	Limited to ovaries	90 percent
II	Pelvic extension	60 to 80 percent
III	Peritoneal implants and/or retroperitoneal or inguinal lymph nodes	20 percent
IV	Distant metastases	Less than 10 percent

Very little is known about this disease and patients often present with few symptoms. Symptoms include non-specific pelvic and abdominal problems, particularly during initial tumour development, making ovarian cancer difficult to diagnose at a low tumour grade [64, 66, 67]. Current initial diagnostic tests, in addition to an examination of the patient's history, include pelvic examination, transvaginal ultrasonography and measuring serum for cancer antigen 125 (CA125). These methods do not provide a specific diagnosis of ovarian cancer, however [64, 67-74], and two thirds of ovarian cancer cases are not diagnosed until the late stages of cancer development (either stage III or IV) resulting in a poor prognosis for the patient [61]. The surgical approach to ovarian cancer is influenced by the stage at diagnosis. If the cancer is found at an early stage, the prognosis for patients is significantly better and fertility-conserving measures may be possible. This may include unilateral salpingo-oophorectomy (removal of the ovary and uterine tube on the affected side), and adjunctive chemotherapy is used if the patient displays residual disease, or has unidentifiable invasive peritoneal implants [63]. As ovarian cancer is often diagnosed at an advanced stage, complete removal of the tumour may not be possible. The patient often requires surgical tumour cytoreduction, followed by a course of combination chemotherapy, using drugs such as carboplatin and paclitaxel [75]. The degree of surgery ranges from unilateral or bilateral salpingo-oophorectomy to total abdominal hysterectomy and removal of pelvic and para-aortic lymph nodes, depending on the stage of cancer progression. These treatment methods are not ideal, as the 5 year survival rate still remains low once the disease has metastasised, dropping to 20% or lower (Table 1.1) [65]. A greater understanding of

the underlying mechanisms of ovarian cancer progression, better prognostic and diagnostic markers for early detection and more effective treatments are urgently needed.

In the ovary, normal function is under the control of an array of hormones and growth factors [76, 77]. Hormones and growth factors play a central role in regulating cell proliferation, differentiation, and apoptosis [78]. Dysregulation of these proteins is likely to be of aetiological importance in ovarian cancer [78, 79]. Some of the hormones proposed to play a role in ovarian cancer pathogenesis include pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), as well as androgens, oestrogens, progesterone and insulin-like growth factors I and II (IGF-I and IGF-II) [80-82]. Gonadotropins [83-86], androgens [84, 87, 88] and oestrogens [84, 89] have all been shown to stimulate cell proliferation of the normal ovarian surface epithelium (OSE), the site of origin of most ovarian cancers. There is, however, recent genetic evidence that the Fallopian tube epithelium may be the site of origin for primary ovarian tumours [90]. It is hypothesised that excessive stimulation of ovarian tissue by pituitary gonadotropins (LH and/or FSH) directly, or indirectly through stimulation of ovarian production of sex steroids, influences malignant transformation of cells [80, 91]. Similarly, excess androgenic stimulation of ovarian epithelial cells has been linked to increased ovarian cancer risk [78, 80] and also to increased proliferation of ovarian cancer cell lines [84, 92-94].

The oestrogens, oestradiol and oestrone, are secreted from granulosa cells of the follicle in the ovary at ovulation, exposing OSE to concentrations that are four times higher than levels in the circulation [80]. Experiments have shown that oestrogens, in addition to stimulating proliferation of OSE cells, can promote formation of serous ovarian cysts [95] and these have been hypothesised to lead to tumour formation [96]. Treatment with oestradiol has been shown to increase vascular endothelial growth factor (VEGF), and cell adhesion in oestrogen receptor (ER) positive cells and increase the migratory potential of ovarian tumours [97]. Progesterone, however, appears to confer a protective effect against ovarian cancer, decreasing proliferation [98], inhibiting invasion [99], suppressing tumourigenesis [100] and inducing apoptosis [101, 102], and higher levels are associated with lower risk of ovarian cancer [78].

IGF-I and -II are potent regulators of ovarian secretory activity, stimulating the release of progesterone, testosterone, oestradiol and other factors [103, 104]. Blocking signalling of the IGF-1 receptor inhibits proliferation in ovarian cancer cell lines [105]. Other growth factors in the ovary may also play a role in the aetiology of ovarian cancer. Proliferation, apoptosis and secretory activity of ovarian cell are influenced by growth factor like epidermal growth factor (EGF) [104], thrombopoietin [106], erythropoietin (EPO) [107], hedgehog protein [108] and their receptors. These influences over ovarian cell function may have significance in ovarian cancer development. Inhibition of the EPO receptor decreases tumourigenesis and invasiveness of ovarian carcinoma cells [109]. The EGF receptor also plays an important role in ovarian cell malignancy, influencing proliferation, survival, adhesion, motility, invasion, and angiogenesis in tumours [110, 111]. Over expression of this receptor has been linked to poor prognosis and decreased therapeutic responsiveness in ovarian cancer patients [110, 111]. Increased vascular endothelial growth factor (VEGF) expression has also been found in ovarian tumours and blocking this receptor can inhibit ovarian carcinogenesis and this shows therapeutic potential [111-113]. Further experimental data is required to establish whether these hormones are driving ovarian cancer development, or whether differences in hormone levels may be a result of the presence of the tumour (through inverse causation bias) [78].

We hypothesise that the ghrelin axis, which includes the peptide hormones ghrelin and obestatin, may play a role in the progression of cancer and may be a useful therapeutic target [15]. Elevated fasting plasma levels of octanoylated ghrelin and obestatin have been described in ovarian cancer patients [114]. In addition, GHSR1a, the ghrelin receptor, is expressed in benign and ovarian cancer tissues [45]. These proteins may provide new targets for the diagnosis and treatment of ovarian cancer.

1.3 The ghrelin axis

1.3.1 Ghrelin, desacyl ghrelin and GOAT

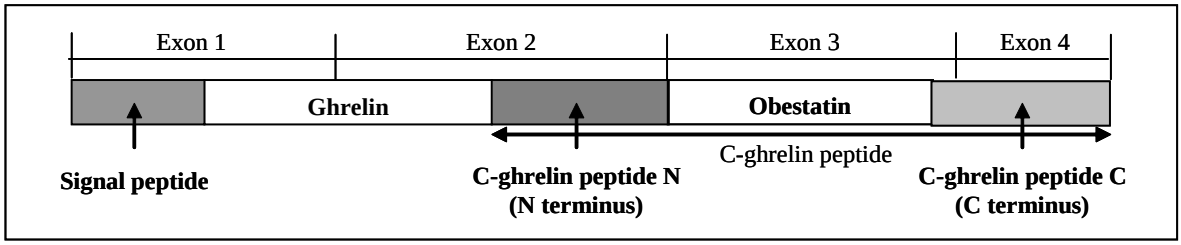
The ghrelin gene (*GHRL*) is located on the chromosomal locus 3p25.3-p26.2 [115]. The gene was originally thought to have a fairly simple structure, with 4 coding exons (exons 1-4), which encode the 117 amino acid preproghrelin peptide, and a

short, 20 bp first exon (Fig. 1.1) [6, 116, 117]. It has more recently been established that the ghrelin gene is complex and contains other upstream exons, (exon -1 and an extended exon 0), as well an extended exon 1 (Fig. 1.1) [116-118]. Research in our group has demonstrated that alternative splicing leads to the production of a large number of mRNA isoforms, including a number of ghrelin antisense transcripts [119]. In humans, parts of exons 1 and 2 of the ghrelin gene code for the ghrelin peptide [116] and exon 3 encodes the peptide hormone obestatin.

The preproghrelin peptide is processed to give a number of peptides, including a 23 amino acid secretion-signal peptide, the 28 amino acid peptide ghrelin and a 66 amino acid C-ghrelin peptide, from which the 23 amino acid peptide obestatin is cleaved [6, 120]. The processing of the preproghrelin peptide to form mature ghrelin and obestatin involves a number of proteins [121]. Cleavage of the ghrelin peptide from preproghrelin is mediated by prohormone convertases (PC) PC1/3, PC2 and furin [122, 123]. The proteins mediating the proteolytic cleavage of obestatin from preproghrelin, remain unclear, although they may include the same prohormone convertases [4, 124].

The major mature form of ghrelin is a 28 amino acid hormone that circulates in two forms, desacyl ghrelin (the major circulating form) and acylated ghrelin (or ghrelin). Ghrelin is octanoylated post-translationally, with the addition of an acyl group to the third amino acid, serine [1, 125-128]. It is the only mammalian peptide known to be post-translationally octanoylated. This unique post-translational modification, which increases the lipophilicity of the molecule [1] (Fig. 1.2), is mediated by the enzyme ghrelin *O*-acyltransferase (GOAT) in the endoplasmic reticulum [2, 3]. In humans, the highest levels of GOAT mRNA expression are in the stomach and pancreas, where it is co-expressed with ghrelin [2, 129]. Lower levels of GOAT transcripts are present in a number of other tissues including brain, colon, oesophagus, fat, heart, small intestines, kidney, liver, lymph node, muscle, pituitary, placenta, salivary gland, testis, thymus, thyroid, uterus [2], adrenal glands [130] and chondrocytes [131] and it is over-expressed in breast tumours and cell lines compared to normal breast [132].

A.



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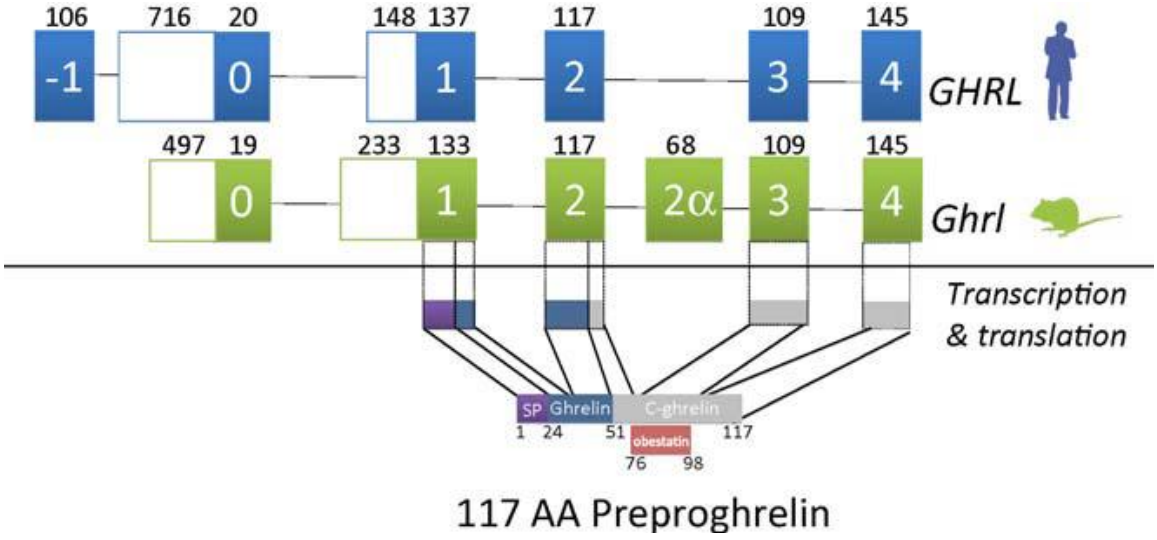

Figure 1.1 The exon structure of the ghrelin gene for the coding of the preproghrelin peptide. (A) The original ghrelin gene structure with 4 exons. (B) Updated genomic organisation of the human and mouse ghrelin genes showing their relationship to the preproghrelin peptide. Exons shaded in white indicate exons that have been found to be extended and the new exon sizes (bp) are shown above each exon [119].

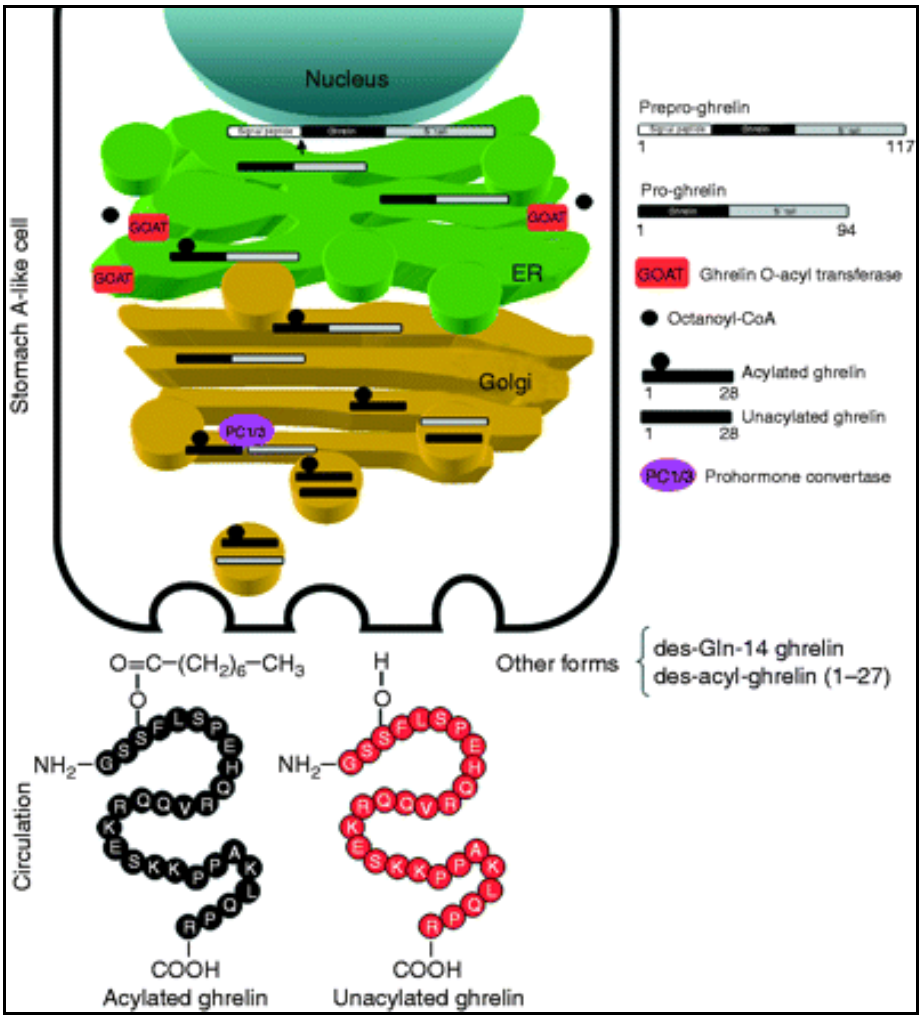

Figure 1.2 Post-translational processing and acylation of the proghrelin peptide. The acylation of proghrelin occurs after the signal sequence is cleaved by a signal peptide peptidase. GOAT is located in the endoplasmic reticulum (ER) and mediates the translocation of the octanoyl-CoA from the cytosolic side of the ER. Once the proghrelin precursor reaches the trans-Golgi compartment, it appears to be cleaved by protein convertases, packaged in vesicles, and released into the blood. Two main forms of ghrelin circulate in the plasma, acylated and desacyl ghrelin, and low levels of other forms also circulate. From Romero *et al.* 2010 [121]

The GOAT enzyme is a member of the membrane-bound *O*-acyl transferase (MBOAT) family [133-135] and it is encoded by the *MBOAT4* gene [2]. It adds the octanoyl group, ($C_7H_{15}CO$), to the serine-3 residue of ghrelin [2, 3]. This fatty acid modification is essential for the activation of its receptor, GHSR1a, and has been hypothesised to assist mature ghrelin in crossing the blood brain barrier via specialised transporters [136, 137]. The N-terminal amino acids, glycine-1, serine-3, and phenylalanine-4 of ghrelin are crucial for recognition by GOAT, and the addition of only two extra amino acids to this N-terminus significantly reduces the ability of GOAT to bind to ghrelin and to attach the octanoyl group [3]. This suggests that the octanoylation of ghrelin occurs after the cleavage of the preproghrelin signal peptide [3]. GOAT is located at the endoplasmic reticulum (ER) and mediates the translocation of the octanoyl-CoA from the cytosolic side of the ER [3]. Although ghrelin is modified by *n*-octanoic acid, GOAT actually has strong preference for *n*-hexanoyl-CoA as an acyl donor [138]. This may account for the observed variations in the length of acyl groups which modify mature ghrelin which have been described. These variant forms, however, are only expressed at low levels. Analogues of ghrelin with acyl chains of 10 (decanoylated) or 11 carbon atoms also exist [139-141] and appear to be more common in other organisms [120, 142-144]. In order to produce octanoylated ghrelin, prohormone convertases and GOAT must be expressed in the cells, and *n*-octanoic acid must be supplied in the culture medium [122].

The ghrelin peptide is unstable in cell culture and likely to have a short half-life, as the octanoyl group is rapidly removed and the ghrelin peptide is proteolytically cleaved [145, 146]. Although a number of des-acylation mechanisms have been hypothesised, the conversion of acylated ghrelin to unacylated ghrelin was recently discovered to be mediated by the enzymatic action of acyl-protein thioesterase 1/lysophospholipase 1 [147]. Desacyl ghrelin, the non-octanoylated form of ghrelin, circulates in plasma at much higher levels than octanoylated ghrelin [1, 125, 126], while other ghrelin analogues circulate at much lower concentrations [120, 148].

Ghrelin is expressed in many cells and tissues throughout the body, however, the major source of circulating ghrelin is the stomach [1, 149] and gastrectomy greatly reduces circulating levels [150]. Lower levels of ghrelin are present in many tissues including the small and large intestine [151], pituitary [152], kidney [153], placenta

[154] and hypothalamus [1] and we have demonstrated ghrelin expression in the ovary, testes, prostate and breast [17, 39, 42, 155]. In the stomach, ghrelin is produced by chromogranin A-immunoreactive X/A-like endocrine cells, or ghrelin-producing cells (Gr cells), located in the mucosal layer of the fundus [151]. These cells lie in close association with capillary networks, allowing transport of secreted ghrelin into the bloodstream for endocrine action [1].

1.3.2 Ghrelin variants and isoforms

Multiple biologically active isoforms of ghrelin exist in addition to full-length, wildtype mature octanoylated ghrelin, and desacyl ghrelin [120]. Other forms of ghrelin include des-Gln14-ghrelin (which is missing the 14th amino acid, glutamine) [120, 148], and ghrelin analogues which are modified by acyl groups of different lengths [120, 126]. Until recently, only two alternatively spliced variants of the wildtype preproghrelin sequences, prepro-des Gln14-ghrelin [120, 148] and exon 3-deleted preproghrelin [17, 156], have been shown experimentally to be translated into peptides. Prepro-des Gln14-ghrelin arises from a three base pair, 5' truncation of exon 2, producing a 116 amino acid preproghrelin peptide and has similar functions to ghrelin [120, 148, 157]. Recently, alternative splicing has been shown to give rise to a number of new variants of the preproghrelin sequence, including obestatin-only transcripts [118]. These variants have the potential to alter the physiological ratio of ghrelin to obestatin, suggesting that alternative splicing is one method of regulation of this gene locus [119]. In addition to producing multiple sense transcripts, the ghrelin gene locus has also been shown to be further complicated by the presence of antisense transcripts, termed ghrelinOS (ghrelin opposite strand) [118]. These transcripts span from an alternative exon -1, with two novel exons (exon 2* and 2**) in intron 2, to an alternative exon 4 of the preproghrelin gene [118]. The physiological significance of these transcripts is yet to be determined.

Exon 3-deleted preproghrelin, as the name suggests, results from the splicing out of exon 3 of the ghrelin transcript [17, 156]. This variant produces a 91 amino acid prepropeptide that, unlike the wildtype and prepro-des Gln14-ghrelin, encodes only ghrelin and does not contain the obestatin-coding region [17, 40, 156]. This causes a frame-shift, which would still allow the synthesis of the mature ghrelin peptide and the production of a novel C-terminal peptide (RPQPTSDRPQALLTSL), the function

of which is unknown [40]. Other novel C-ghrelin and obestatin-only transcripts have also recently been discovered, however, the function of these peptide has not been widely studied [118].

1.3.3 The growth hormone secretagogue receptor (GHSR)

Ghrelin acts through the cognate ghrelin receptor, the growth hormone secretagogue receptor (GHSR) [1]. The GHSR is a highly conserved, seven transmembrane domain G protein coupled receptor (GPCR) [158]. GHSR was originally cloned from the pituitary and hypothalamus of humans and swine [159]. It was discovered as the target for synthetic growth hormone secretagogues (GHS), a class of peptide and non-peptide compounds which stimulate the release of growth hormone (GH) from the anterior pituitary [159]. The GHSR gene consists of two exons, with exon 1 encoding transmembrane domains I-V and exon 2 encodes transmembrane domains VI and VII [160]. The exons are separated by a single 2 kb intron (Fig. 1.3). There are two known GHSR mRNA isoforms, GHSR1a and GHSR1b. The GHSR type 1a transcript, encodes the full-length, functional 366 amino acid receptor (Fig. 1.3) [160]. Activation of the GHSR1a in the pituitary leads to the release of intracellular calcium, which is the trigger for growth hormone (GH) release [160]. It is widely shown to be the mediator of appetite, GH-releasing activity, and a range of

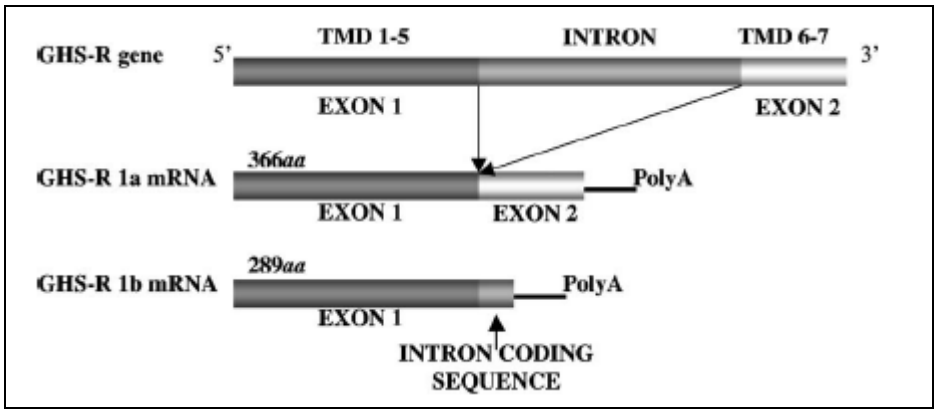

Figure 1.3 A representation of the human growth hormone secretagogue receptor (*GHSR*) gene and the GHSR 1a and GHSR 1b mRNA isoforms. The GHSR1b isoform has putative alternative polyadenylation and splice sites and contains intron-coding sequence. TMD: transmembrane domains. Adapted from Jeffery *et al.* 2003 [156].

cardiovascular and metabolic functions of ghrelin [4]. This receptor has strong constitutive activity (in the absence of ligand), which is 50% of its maximal activity, [161-165] and this high level of activity is comparable to that of the ORF74 receptor oncogene, encoded by human herpes virus 8 [166, 167].

Ghrelin was initially demonstrated to stimulate and increase intracellular calcium in *Xenopus* oocytes, acting through the GHSR [1, 159]. Ghrelin has a wide range of physiological effects, and has been demonstrated to stimulate signalling through a number of different pathways (Fig. 1.4). The mitogen activated protein kinase (MAPK)/extracellular signal-regulated kinase 1/2 (ERK1/2) pathway has been shown to mediate a number of the functions of ghrelin (Fig. 1.4), including ghrelin-stimulated cell proliferation [25, 40, 57, 168-172], regulation of inflammation [173], angiogenesis [171, 172, 174], inhibition of apoptosis [175-180] and cell migration [171, 172, 174]. It has also been reported that ghrelin signals through the Akt/phosphatidylinositol-3-kinase (PI3K) pathway to regulate proliferation [25, 168, 169], angiogenesis [172], cell migration and invasion [54, 172], to inhibit apoptosis [175-183], and to influence metabolism [184].

GHSR1b is a truncated transcript, resulting from the inclusion of intronic sequence in the mRNA, and it produces a truncated, 289 amino acid peptide (Fig. 1.3). The inclusion of intronic sequence leads to the introduction of a premature stop codon, and a novel 24 amino acid C-terminus is encoded after the 5^{th} transmembrane domain of the GHSR [185]. This receptor isoform was believed to be biologically inactive, however, there is evidence that it may regulate the expression of the GHSR1a at the cell surface [186]. Co-expression studies of GHSR1a and GHSR1b demonstrated that dimerisation of these two receptors reduces constitutive GHSR1a signalling [162, 186]. This reduces GHSR1a-stimulated activation of phosphatidylinositol-specific phospholipase C (PI-PLC) activity, leaving ghrelin-dependent signalling, such as extracellular signal-regulated kinases 1/2 (ERK1/2) activation, unaffected [162, 186]. This inhibition of constitutive signalling is thought to be due to the translocation of GHSR1a to the nucleus as a result of the formation of GHSR1a/GHSR1b heterodimers [186].

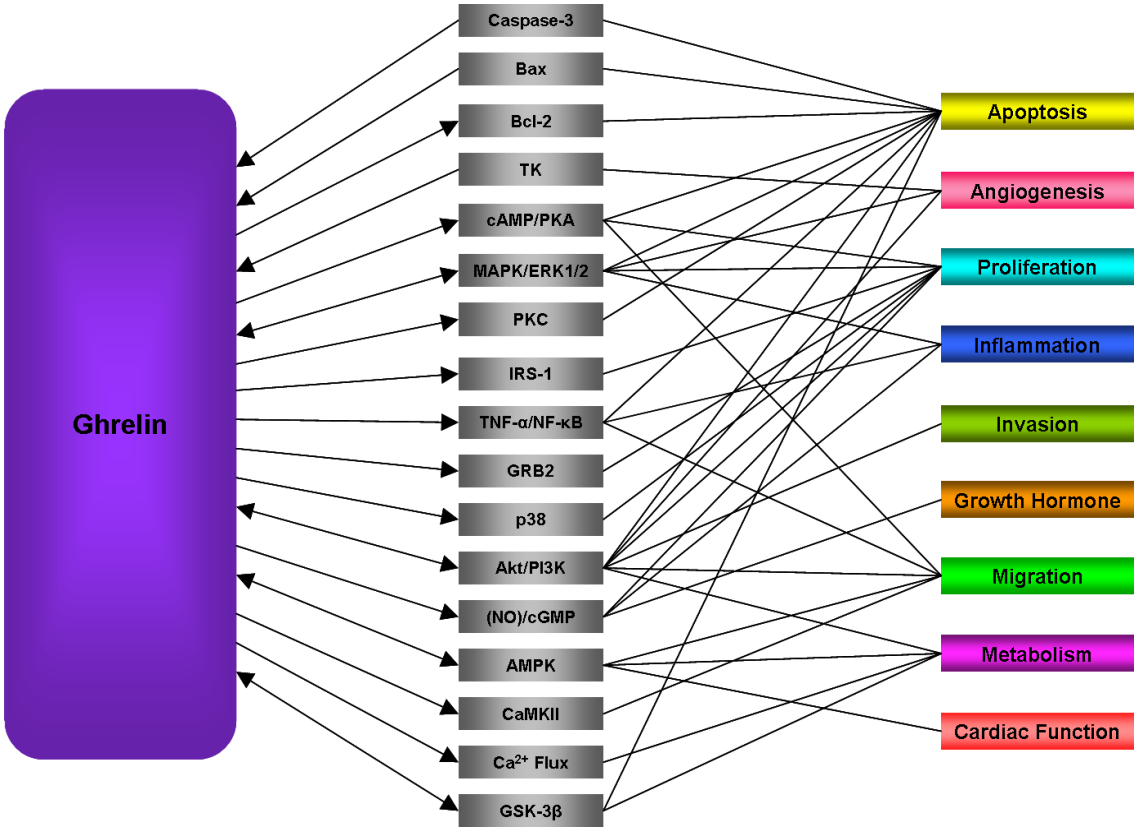

Figure 1.4 An overview of ghrelin signalling pathways, (other than those involved in appetite and gastrointestinal functions), and the functions that they mediate. The diagram shows whether ghrelin activates (→) or inhibits (←) signalling pathways, or whether both activation and inhibition have been described (↔) and these are linked to the observed physiological effects that are mediated by ghrelin signalling [16, 25, 40, 47, 52, 54, 55, 57, 168-184, 187-194].

1.3.4 The alternative ghrelin receptor and desacyl ghrelin

In addition to the GHSR, there is strong evidence that an unidentified alternative ghrelin receptor exists. Ghrelin has physiological effects in cell types that do not express the GHSR1a [195, 196]. Desacyl ghrelin circulates in plasma in far higher concentrations than ghrelin (representing approximately 80% of total circulating ghrelin) [126, 151], and it does not bind or activate GHSR1a [1, 126]. It has become evident that desacyl ghrelin also has physiological effects, which are independent of the GHSR1a. Some of the actions of ghrelin, including GH release [1, 197-200], and the regulation of appetite require ghrelin to be octanoylated, however. The role of desacyl ghrelin in feeding is controversial [201], and a number of studies have suggested that desacyl ghrelin may suppress appetite [202-204].

Ghrelin and desacyl ghrelin have similar or identical actions in a number of cell and tissue types (Table 1.2). For example, in rat pregnancy, both peptide variants (ghrelin and desacyl ghrelin) bind to foetal tissues and they lead to increased Ca^{2+} influx in discrete cultured foetal skin cells [205]. They also promote rat bone marrow adipogenesis through mechanisms which are independent of GSHR1a [206]. Desacyl ghrelin displays comparable cardioprotective effects to ghrelin [157, 178, 207-209], and both forms inhibit apoptosis in primary adult and H9c2 cardiomyocytes and endothelial cells [178]. The H9c2 cell line does not express the GHSR1a and these functions, therefore, must be mediated by the alternative ghrelin receptor [178]. In the H345 small cell lung carcinoma (SCLC) cell line, both forms of ghrelin increase apoptosis [28]. Ghrelin and desacyl ghrelin also display other similar cellular functions. Ghrelin and desacyl ghrelin both increase proliferation in human osteoblasts, which do not express GHS-R1a [168], while both forms of ghrelin decrease proliferation in the CALU-1 small cell lung carcinoma (SCLC) cell line [28]. Although this list of studies is not exhaustive it demonstrates that both forms of ghrelin are likely to act via an alternative receptor [210].

As ghrelin and desacyl ghrelin can both regulate proliferation, apoptosis, adipogenesis and cardioprotective effects, often in the absence of the GHSR1a, it is likely that they are acting through the alternative ghrelin receptor [210]. Ghrelin is known to function in a number of cell types in the absence of GHSR1a [50, 195, 206]. Ghrelin binding sites have been demonstrated in normal thyroid glands, thyroid

Table 1.2 Summary and comparison of ghrelin gene derived products effects.

Adapted from Soares *et al.* 2008 [4].

Effect	Ghrelin	Des-acyl ghrelin	Obestatin
Endocrine			
GH	↑	↔↓	↔↓
ACTH, TSH, AVP	↑	↔	↔
Appetite/body weight	↑	↓↑	↓↔
Water intake	↓	↔	↓
Metabolism			
Glucose			
Glucose sensing	↓	nd	nd
Insulin secretion	↓↑	↓	↓↑↔
Insulin action	↑ (periphery)/↓ (liver)	↔ (periphery)/↓ (liver)	nd
Glucose production	↑	↓	nd
Lipid content			
Liver	↑	nd	nd
Muscle	↓	nd	nd
Adipocyte	↑	↑	nd
Reproduction			
LH secretion	↓	↓	nd
Embryogenesis	↓	nd	nd
Testosterone secretion	↓	nd	nd
Spermatogenesis	↓	nd	nd
Follicle survival and action	↑	nd	↑
Gastrointestinal			
Exocrine secretion	↑↓↔ (stomach)/↑ (pancreas)	↔ (stomach)	↑ (pancreas)
Epithelial protection	↑	nd	nd
Motility	↑ (stomach and colon)	↓ (stomach)/↔ (jejune)	↓ (stomach and jejune)/↔
Cardiovascular			
Vascular dynamics			
Macrovessels dilation	↑ (systemic)/↓ (coronary)	↑ (systemic)	nd
Microvessels dilation	↑	nd	nd
Endothelium function	↑	nd	nd
Heart function	↑	↑	↔
Cell proliferation	↑↓	↑↓	↑
Immune function			
Immune cell production	↑	↔	nd
Cytokine production	↓	↔	nd
Neutrophil action	↓	nd	nd
Bone			
Osteoblast production	↑	↑	↔
Osteoblast action	↑	nd	↔
Sleep	↑	↔	↑
Memory	↑	↔	↑
Anxiety	↑	↔	↓
Iris muscle relaxation			
Sphincter	↑	↑	nd
Dilator	↑	↔	nd

(↑), stimulation; (↓), inhibition; (↔), no effect; nd, effect not determined.

tumours, and thyroid cancer cell lines, in the absence of the specific GHSR mRNA [44]. GHSR1a expression is also absent in the HepG2 cell line [195], although ghrelin stimulates cell signalling and cell proliferation in this cell line [147, 195, 211]. Similarly, ghrelin stimulates cell proliferation in the HEL human erythroleukemic cell line, although GHSR1a is undetectable [50].

1.3.5 Ghrelin function

Ghrelin has a wide array of functions [119] (Fig. 1.5), including roles as a growth hormone secretagogue and in regulating appetite and energy balance [1]. Since its isolation and identification in 1999, ghrelin has proven to be a multifunctional peptide, with roles in regulating the immune system, metabolism, reproduction, sleep, memory and anxiety [4]. It also appears to have roles in regulating the cardiovascular system and bone, and in regulating cell proliferation, apoptosis, migration and angiogenesis [4].

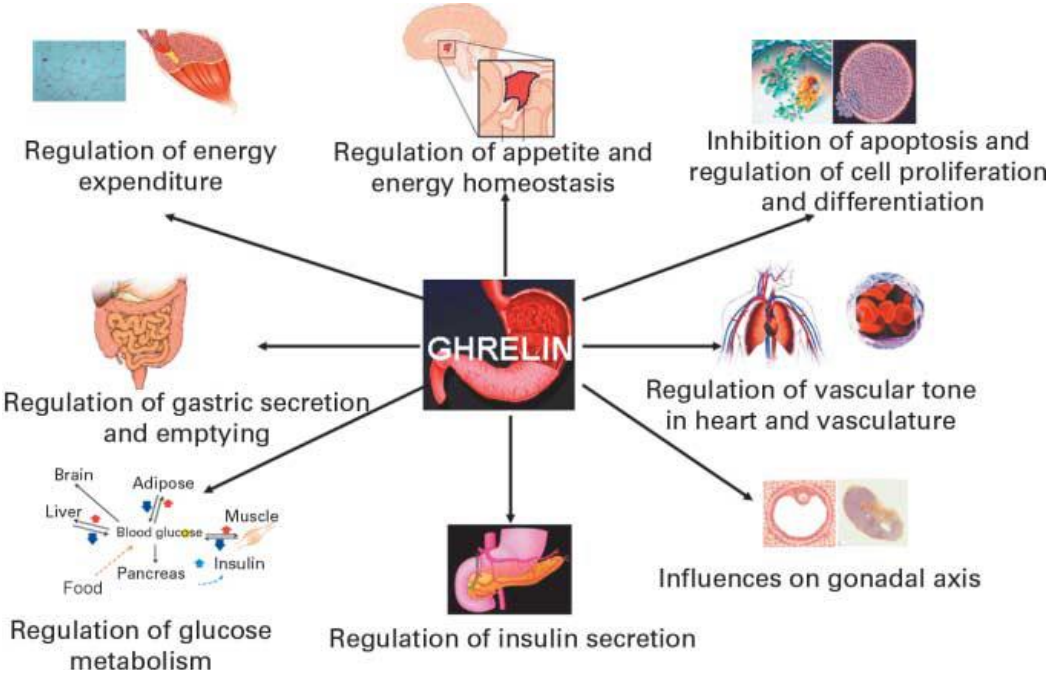

Figure 1.5 A diagram illustrating the diversity of ghrelin functions. Ghrelin is highly expressed in the stomach and at lower levels in many other tissues. Its effects on appetite, gastric activity and energy metabolism are mediated via the hypothalamus, brainstem and afferent vagal nerve fibres. Ghrelin is produced in lower amounts in other tissues where it may have a number of direct effects, including the regulation of apoptosis, cell proliferation and cardiovascular and immunological function. Adapted from Gil-Campos *et al*., [212].

1.3.5.1 Ghrelin and growth hormone (GH) release

Ghrelin was originally discovered as the endogenous ligand for the GHSR, based on its ability to act as a growth hormone secretagogue [1]. Acting at the pituitary and hypothalamic levels, it stimulates the release of growth hormone (GH) from the pituitary both *in vitro* and *in vivo* in a range of species including humans [1, 197-199]. This is a dose-dependent response, mediated through the GHSR1a which is expressed by somatotroph cells in the anterior pituitary [200]. It induces activation of growth hormone releasing hormone (GHRH) neurons and inhibits somatostatin neurons in the hypothalamus [213]. This function is not shared by desacyl ghrelin, as it is unable to activate GHSR1a, or stimulate GH release. Ghrelin also stimulates the release of adrenocorticotrophic hormone (ACTH) and prolactin (PRL), albeit to a much lesser extent, from the anterior pituitary, and antidiuretic hormone (ADH) release from the posterior pituitary and it inhibits the pituitary-gonadal axis [214-220].

1.3.5.2 Ghrelin in appetite regulation, energy balance and metabolism

Ghrelin plays a role in regulating energy homeostasis [221] and promotes adipogenesis [222]. It originates from the stomach, and it induces adiposity and provides information about nutrient availability to the brain [1, 223]. Ghrelin octanoylation appears to be essential for these functions [201]. Acylated ghrelin increases food intake, gut motility and weight gain in humans [224] and it increases gastric acid secretion in rats [225], as well as reducing energy utilisation and inhibiting leptin-induced feeding reduction [223, 226]. Plasma ghrelin levels rise during fasting (or prior to meals) and fall after feeding [149, 227-229]. The effects on appetite are believed to be independent of its effects on bodyweight and adiposity [222, 223, 230]. Ghrelin contributes to the regulation of body weight, adjusting levels in response to weight gain and loss [231]. Fasting circulating ghrelin concentrations correlate inversely with body weight (and body mass index) and levels increase after weight loss [232]. The chronic administration of ghrelin promotes weight gain and adiposity [221]. Notably this weight gain is not driven solely by food intake, ghrelin also influences preference towards dietary fat [221].

Ghrelin regulates lipid metabolism in the liver, skeletal muscle and adipose tissue [233]. Both ghrelin and desacyl ghrelin promote lipogenesis [206] and inhibit

isoproterenol (isoprenaline)-induced lipolysis [196]. Ghrelin increases triglyceride deposits in the liver, inhibiting fatty acid oxidation, and decreases triglyceride deposition in muscle, increasing mitochondrial oxidative enzyme activity and fat metabolism [233].

Ghrelin exerts its orexigenic actions by activating neuropeptide Y (NPY), agouti related peptide (AgRP) and oxytocin neurons in the arcuate nucleus of the hypothalamus, while neurones expressing the anorexigenic neurotransmitters, cocaine and amphetamine related transcript (CART) and pro-opiomelanocortin (POMC), are inhibited [234, 235]. The hypothesised mechanism of the orexigenic effects of ghrelin that are mediated by GHSR1a and are highly complex (Fig. 1.6). This pathway involves activation of the GHSR1a, which induces Ca^{2+} flux, and phosphorylation and the sequential activation of a number of signalling molecules including calmodulin kinase kinase 2 (CaMKK2) and AMP-activated protein kinase (AMPK) [236, 237].

The role of desacyl ghrelin in regulating energy homeostasis and food intake is more controversial. It is clear that acylated ghrelin has a strong stimulatory effect on appetite, mediated by GHSR1a. As GHSR1a mediates the effects of ghrelin on appetite, and desacyl ghrelin does not signal through this receptor, it is unsurprising that a number of reports have demonstrated that it has no effect on feeding [201, 238]. A number of other studies, however, have shown that desacyl ghrelin may have a suppressive effect on appetite [202-204].

Ghrelin may also play a part in water consumption. Centrally and peripherally administered ghrelin has been shown to potently inhibit water intake in rats [239, 240] and this function is not shared by desacyl ghrelin [196, 241].

1.3.5.3 Ghrelin and the regulation of blood glucose and insulin release

Ghrelin has been implicated in glucose homeostasis [242], although its role is currently controversial. Ghrelin appears to have a modulatory effect on glucose-sensing neurons in the dorsal vagal complex [243, 244], and may also regulate insulin secretion [245-247] and hepatic glucose production [25, 248]. Systemic administration of ghrelin, in most cases, has been shown to result in the inhibition of

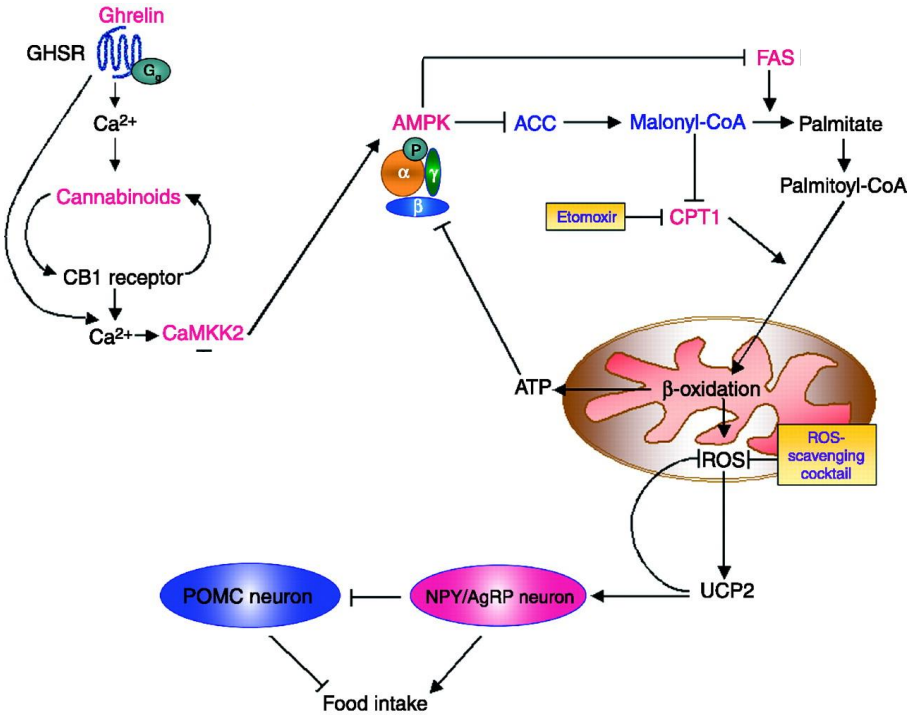

Figure 1.6 A diagram showing a simplified representation of the molecules which comprise the ghrelin signalling cascade in the appetite-inducing effect of ghrelin. The activation of signalling molecules (highlighted in pink) leads to increased food intake, whereas the activation of inhibiting molecules (highlighted in blue) would lead to inhibition of food intake. It is hypothesized that ghrelin, through the activation of GHSR1a, induces Ca^{2+} flux, which induces the phosphorylation and activation of calmodulin kinase kinase 2 (CaMKK2), either with or without the prior induction of endocannabinoids activating cannabinoid receptor type 1 (CB1). This leads to a secondary Ca^{2+} flux. CaMKK2 then activates AMP-activated protein kinase (AMPK) which in turn phosphorylates and inhibits acetyl-CoA carboxylase (ACC), preventing the production of malonyl-CoA. This allows carnitine palmitoyl transferase 1 (CPT1) and fatty acids to be transported into the mitochondria, facilitating mitochondrial fatty acid oxidation. These then undergo mitochondrial β-oxidation, producing reactive oxygen species (ROS), and promoting expression of uncoupling protein 2 (UCP2), which buffers the production of ROS. UCP2 stimulates hypothalamic mitochondrial respiration, mediating the activation of NPY/AgRP neurons, stimulating food intake and inhibiting POMC neurons that inhibit food intake. From Kola *et.al* 2009 [236].

insulin release [245, 246, 249, 250]. Furthermore, ghrelin also stimulates insulin-induced glucose uptake in adipocytes [251]. Intriguingly, physiological concentrations of glucose or insulin were shown to have little effect on plasma ghrelin levels [252]. This function of ghrelin appears to be dependent upon octanoylated ghrelin, as GOAT knockout mice, placed on 60% calorie restricted diet, became moribund with hypoglycaemia and were rescued by the injection of acylated ghrelin or GH [253]. This study has recently been refuted, however, in studies using knockout mice subjected to prolonged 60% calorie restriction, which have shown that GOAT and the ghrelin axis are not required for survival during caloric restriction [254]. Blood glucose levels, however, were not measured at the same time in these two studies, ½ hour prior to feeding versus 8 hours prior in the later study. Additionally, these studies were in different strains of mice.

1.3.5.4 Gastrointestinal effects of ghrelin

Ghrelin increases gut motility [225], and acting independently of the hormone motilin, it accelerates gastric emptying and colonic motility [255-258]. Ghrelin stimulates the secretion of pancreatic fluid and secreted proteins [259, 260]. Interestingly, desacyl ghrelin inhibits gastric emptying without influencing the small intestine, or altering gastric secretions [204, 261-263]. It is currently unclear whether ghrelin affects gastric acid secretion, as different studies have reported that ghrelin stimulates [225, 264], inhibits [263] or has no effect [262] on acid secretion.

1.3.5.5 The role of ghrelin in the normal immune system

Ghrelin has immunoregulatory functions [265] and is expressed in a number of cell types in the immune system including human T cells, B cells, and neutrophils [266], however, its exact role is yet to be defined. In human monocytes and T lymphocytes, ghrelin, acting through the GHSR, inhibits the expression of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α [267, 268]. In murine macrophages, pre-treatment with ghrelin resulted in inhibition of lipopolysaccharide (LPS)-induced pro-inflammatory cytokines, IL-1β and TNF-α, and it decreased nuclear factor-kappa B (NFκB) activation, a transcription factor that plays important roles in the immune system [269]. In response to ghrelin treatment, LPS-stimulated murine macrophages significantly increased the release of the anti-inflammatory cytokine, IL-10, in a dose-dependent and time-dependent manner [269]. Ghrelin also inhibits leptin-

induced cytokine expression in a dose-dependent manner [267], suggesting a link between the metabolic axis and the immune system. These studies show potential for a significant role for ghrelin in inflammation.

These immune responses may be limited to octanoylated ghrelin and may not be shared by desacyl ghrelin [268-270]. This however, may be challenged by recent studies that have shown that ghrelin induced the release of histamine from rat peritoneal mast cells in the absence of GHSR1a, and this was likely to occur through a receptor-independent mechanism [271].

1.3.5.6 The role of ghrelin in the cardiovascular system

Ghrelin also has regulatory roles in the cardiovascular system [272, 273]. The intravenous administration of ghrelin decreases arterial blood pressure without affecting heart rate [274, 275], and it increases cardiac index (cardiac output divided by the body surface area in square meters) and stroke volume index (obtained by dividing cardiac index by heart rate) [215, 276]. Intracoronary infusion of ghrelin caused coronary vasoconstriction through the inhibition of a vasodilatory β_2-adrenergic receptor-mediated effect related to a release of nitric oxide [277]. Similarly, in another study ghrelin acutely stimulated increased production of nitric oxide in bovine aortic endothelial cells (BAEC) in primary culture in a time- and dose-dependent manner, using a signalling pathway that involves GHSR1a, PI3K, Akt, and endothelial nitric oxide synthase (eNOS) [278].

In vitro ghrelin also has a range of effects on cardiomyocytes and endothelial cells. Ghrelin can increase AMPK activity in heart muscle and modulate intracellular energy balance in a cell-specific manner [192]. In isolated rat ventricular myocytes ghrelin and the synthetic growth hormone secretagogue, hexarelin, increase the amplitude of intracellular T-type and the L-type Ca^{2+} currents [279]. They decrease in the transient outward potassium current (I_{to}) and lead to subsequent prolongation of action potential duration [280] through the activation of GHSR1a and PKC [279, 280], as well as phospholipase C for the decreased I_{to} current [280]. Furthermore, in cardiomyocytes ghrelin has been reported to inhibit apoptosis [178, 281], promote cell survival by modification of MAPK pathways and activation of GHSR1a [281], decrease inotropy (force of contraction) [157, 282] and lusitropy (relaxation) [282]

and improve myocardial function during ischemia, or myocardial injury [209, 281, 283, 284]. In endothelial cells, ghrelin stimulates angiogenesis [172] and inhibits pro-inflammatory cytokine production [268]. Ghrelin also inhibits angiotensin II-induced migration [190] and stimulates proliferation [169] in aortic endothelial cells, whereas in aortic smooth muscle cells it inhibits angiotensin II-induced proliferation and smooth muscle contraction [285].

Desacyl ghrelin has similar effects to ghrelin in cardiac function. It displayed comparable potency as an endothelium independent vasodilator [207, 208], and decreased inotropy [157] and inhibited cardiomyotcyte apoptosis [178]. It also retains the cardioprotective effect observed with myocardial injury [209].

1.3.5.7 The role of the ghrelin axis in reproduction

Numerous studies suggest the ghrelin has a wide array of effects in reproduction [4, 286]. Ghrelin is expressed in a number of reproductive organs including the placenta [154], ovary [155, 287-291], endometrium [292, 293] and Leydig cells of the testes [42, 294-296]. Ghrelin expression has also been demonstrated in embryos [297, 298]. There is increasing evidence that ghrelin can suppress a number of hormones involved in the gonadal axis, including gonadotropin-releasing hormone (GnRH) [299-301], luteinizing hormone (LH) [302-306], and follicle stimulating hormone (FSH) [304, 305], in both males and females in humans, rats and other higher mammals. It is interesting that these effects on LH and FSH appear to be attenuated in females during the menstrual cycle [307] and also in males when ghrelin is administered in combination with GnRH [305].

Ghrelin is expressed in female reproductive organs. It exerts an inhibitory effect on steroidogensis (lowering progesterone and oestradiol production) in the ovaries [308, 309]. Ghrelin levels have been shown to fluctuate throughout pregnancy [310, 311], peaking at 18 weeks of pregnancy [310, 311], and it may contribute to weight gain during pregnancy [312]. In a study of mice, ghrelin may have an inhibitory effect on the early development of the embryo [298]. Porcine studies suggest that this may occur through an inhibition of the organisation of microtubules and microfilaments in the oocytes [313], or by having a negative effect on the structural integrity of blastocysts [314]. Ghrelin also appears to play further roles in foetal development,

playing a regulatory role in the late stages of pregnancy, and increasing birth weight, irrespective of a restricted maternal diet [205]. Umbilical-cord ghrelin levels exceed that of the mother [315] and high levels of desacyl ghrelin are present in foetal blood and amniotic fluid [205]. Both desacyl ghrelin and octanoylated ghrelin have binding sites in foetal tissues and increase Ca^{2+} influx in discrete cultured foetal skin cells [205]. Additionally, high levels of the ghrelin receptor (GHSR) have been found throughout peripheral tissues of foetal rat from embryonic day 14 until birth [205]. Postnatally, ghrelin is secreted into the colostrum and milk [316] and lactation induces the production of GHSR in both the hypothalamus and pituitary [317, 318].

1.3.5.8 The role of ghrelin in bone

Ghrelin and its receptor have been identified in osteoblasts, which are mononuclear cells that form bone. In osteoblasts, ghrelin directly stimulates proliferation, DNA synthesis and differentiation, and inhibits apoptosis [168, 189, 319-321]. Ghrelin significantly increases levels of the bone formation markers, alkaline phosphatase (AP) and osteocalcin (OC), as well as increasing calcium accumulation in the bone matrix [320, 322, 323]. As a result, ghrelin is believed to stimulate osteogenesis (bone formation) in intramembranous bone growth and improves the repair of calvarial bone defects in rats [324].

It is unclear whether ghrelin has a direct impact on bone mineral density (BMD), as some studies have found that it directly increases BMD in rats [322] and others have found no effect in mice [325]. In a study of obese and healthy control children, it was concluded that the effect of ghrelin on BMD is mediated by specific body composition parameters (lean and fat mass) [326]. In another study in adult humans no association was seen between ghrelin and BMD [327].

Interestingly, ghrelin has also been identified in teeth, a tissue that shares many similarities with bone, and its potential physiological roles are being investigated [328]. Ghrelin is also expressed in cartilage, where it has been identified as a potential chondrocyte growth factor [323]. While there have been few studies into the role of desacyl ghrelin, like ghrelin, it stimulates human osteoblast proliferation [168]. This is thought to be independent of GHSR1a, as these cells do not appear to express the GHSR1a mRNA transcript [168].

1.3.5.9 The effect of ghrelin on sleep, memory, learning and behaviour

Ghrelin has several effects on behaviour and higher order brain functions. It promotes slow-wave sleep in humans [329], and it has been used to modify sleep-wake patterns in rats [330], as well as increasing non-rapid eye movement sleep (NREMS) [331]. In addition, ghrelin improves cognition, spatial learning and memory [332, 333] and ghrelin facilitates place-learning linked memory processes, acting in the amygdala [334]. Ghrelin increases memory retention in rats [335], however, it improves long-term memory, rather than short-term memory [336], suggesting that ghrelin is involved in the acquisition of memories rather than memory retrieval [336]. Based on these observations, the central ghrelin receptor could be considered as a new drug target to treat diseases affecting cognition, or to enhance learning and memory processes [332, 337].

Ghrelin is also believed to mediate behavioural responses to stress, as circulating ghrelin levels rise following stressful stimuli [338, 339]. Studies on the relationship between ghrelin and mood are limited and reports are conflicting regarding whether or not ghrelin has an anti-depressant effect [338, 340]. Similarly, several studies suggest that ghrelin has an anxiolytic effect [338, 339, 341], whereas several others suggest that it has an anxiogenic effect [335, 342, 343].

1.3.6 Ghrelin in disease

Ghrelin has been implicated in a number of diseases and disease processes. As ghrelin potently stimulates appetite, and has a role in energy balance and body composition, it has been a major focus of obesity research [223, 228, 344-347]. Ghrelin dysregulation is also associated with other appetite-related conditions, including the eating disorders, anorexia nervosa (typified by high ghrelin levels and insensitivity) [228, 348, 349] and bulimia nervosa (with high plasma ghrelin levels) [350-352]. It is unclear in these disorders whether ghrelin levels are influenced by the binge/purging process, or by the shortcomings in energy status [353], however, plasma ghrelin levels are generally strongly inversely correlated with body mass index (BMI) [353]. This is not the case in Prader-Will syndrome, however, where patients show high ghrelin levels, hyperphagia and adiposity [354-356]. As ghrelin plays a role in appetite and energy homeostasis, adiposity and glucose balance, it is also likely be involved in metabolic diseases including diabetes mellitus type II [357,

358] and metabolic syndrome [357].

As ghrelin promotes feeding and positive energy balance, the ghrelin axis is useful as a treatment target for diseases associated with anorexia and chronic wasting (cachexia), including anorexia nervosa [359, 360], chronic kidney disease [361], chronic heart failure [362] and cancer. Ghrelin has also been reported to affect several gastrointestinal diseases such as inflammatory bowel disease, coeliac disease, infectious diseases, functional disorders and diabetic gastroenteropathy [363].

Ghrelin also exerts many cardioprotective effects, including reduced peripheral resistance, and improved heart contractility and cardiac output, suggesting a beneficial effect in cardiovascular disorders [364].

Ghrelin has been associated with polycystic ovary syndrome (PCOS). PCOS is characterized by chronic anovulation and hyperandrogenism, with symptoms including irregular menses or infertility, and cutaneous manifestations, including hirsutism, acne, or male-pattern hair loss [365]. There is some ambiguity over the levels of ghrelin in PCOS, with some studies showing ghrelin levels are decreased [366-370], unchanged [371-373], or elevated in PCOS women [374]. Ghrelin appears to be involved in secondary conditions that develop with PCOS. Ghrelin levels were highly correlated to the degree of insulin resistance in one study [366] and contributed to the abnormal hormonal pattern seen in PCOS in another [371]. It may also play a role in the development of obesity, hyperinsulinemia and insulin resistance seen with metabolic syndrome associated with PCOS [372]. The injection of ghrelin seems to over-ride the defect in GH secretion in obese women with PCOS and induces changes in glucose and insulin regulation in both control and obese patients with PCOS [375]. A number of these diseases have been linked to increased cancer risk. Metabolic syndrome [376], obesity [377], inflammatory bowel disease [378] and PCOS [379] have been shown to have a significant impact on the incidence and/or progression of cancer.

1.3.7 Ghrelin in cancer

1.3.7.1 Expression of the ghrelin axis in cancer

Ghrelin and the ghrelin axis have been associated with cancer progression. Ghrelin and the GHSR1a and GHSR1b isoforms are expressed in a wide array of cancer tissues (Table 1.3). In many cases, the expression of ghrelin and GHSR are different in cancer tissues compared to normal tissue. Ghrelin expression is increased in astrocytoma [16], breast cancer [17], thyroid cancer [43, 44] lung cancer [26] and ovarian cancer [380] and down-regulated, if not absent, in others, including stomach cancer [19, 22, 23], pituitary adenomas [32, 33], renal cancer [41], testicular cancer [42] and oesophagogastric adenocarcinomas [21]. In a number of tumours, including pancreatic endocrine tumours [31], pituitary adenomas [32, 33], prostate cancer [38, 39] and breast cancer [17], ghrelin is co-expressed with its receptor and ghrelin could, therefore, play an autocrine role in these tumours. In a number of cancers, however, GHSR1a expression is down regulated or absent, while the non-functional, truncated form of the receptor, GHSR1b, is expressed, or up-regulated, compared to normal tissue [18, 27, 29, 48, 53].

Some studies, using small numbers of subjects, have investigated the levels of plasma ghrelin and demonstrated a relationship with cancer [381]. Plasma ghrelin levels have been shown to be elevated in ovarian cancer [114], non-small cell lung cancer [382], uterine leiomyoma [383] and prostate cancer [384] and decreased levels have been demonstrated in colorectal cancer [385] and liver cancer [386]. This increase, however, in not necessarily linked with an increase in total plasma ghrelin [114, 384, 387]. Many of these studies suggest that ghrelin has potential as a biomarker, however, to determine its efficacy larger studies need to be performed. It is likely that ghrelin levels are more directly correlated with nutritional status rather than cancer itself, and ghrelin levels are elevated in cachexia [381].

1.3.7.2 Single nucleotide polymorphisms (SNPs) and epigenetic regulation (hypermethylation) in the ghrelin axis and cancer

A number of single nucleotide polymorphisms (SNPs) in the ghrelin (*GHRL*) and *GHSR* genes have been linked to altered cancer risk. SNPs have been linked in breast cancer to tumour development [388]. A 20% increase in breast cancer risk was

Table 1.3 Protein and mRNA expression of ghrelin and the GHSR in tumour tissues and cell lines. (-) no expression, (++) is expressed or has moderate expression, (+) low expression, (+++) high expression, ↑ higher than normal tissue levels, ↓ lower than normal tissue levels.

Cancer Type	Ghrelin			GHSR				
	mRNA	Protein	References	1a mRNA	1b mRNA	1a Protein	1b Protein	References
Adrenal Tumours								
Phaeochromocytoma	(+)		[56]					
Adrenocortical adenoma	(+)		[56]					
Non-functioning adrenocortical adenoma	(+++)↑		[53]	(-)↓	(++)↑			[53]
Cortisol-producing adenomas	(++)↑		[53]	(++)↑	(+++)↑			[53]
Aldosterone-producing adenoma	(++)↑		[53]	(-)	(+)↑			[53]
Adrenocortical carcinoma	(+)		[53]	(-)	(++)↑			[53]
Adrenocortical carcinoma cell lines (NCI-H295, SW13)	(+)		[53]	(+)	(+++)			[53]
Astrocytoma								
Low grade tumours		(+) (+++)	[16]			(+) (+++)		[16]
High grade tumours		(+) (+++)	[16]			(+) (+++)		[16]
Astrocytoma cell lines (U-118, U-87, CCFSTTG1, SW1008)						(+++)		[16]
Breast Cancer								
Tumour		(++)↑	[17]			(++)	(++)	[17]
Breast cancer cell lines (MDA-MB 231, MCF7) (T47D) (MDA-MB 435)	 (++) (++) (++)		 [17] [17] [17]	 (+)(-) (+)(-) (++)	 (++) (+) (++)			 [17, 18] [17, 18] [17]
Endometrial Cancer								
Endometrial cancer	(++)	(+)(-)↓	[46, 49]	(+)	(+)			[49]
Endometrial cancer cell lines (HEC1B, KLE)	(+)	(+)	[49]	(+)	(+)	(+)		[49]
Gastrointestinal Tract Cancer								
Oral squamous cell carcinoma		(+)(-)↓	[24]					
Oesophagogastric adenocarcinomas	(-)	(-)	[21]	(++)↑				[20]
Stomach	(+++)	(+++)↓↓ (++)	[19, 22, 23, 56]	(-) (++)↑	(-)			[19, 20]
Gastric adenocarcinoma		(-)	[22]					
Duodenum	(-)	(++)	[19]	(-)	(-)			[19]
Ileum	(++)	(-)(++)	[19]	(++)	(++)			[19]
Appendix	(-)	(++)	[19]	(-)(++)	(-)			[19]
Appendix- Well-differentiated neuroendocrine tumour		(-)	[56]					
Colon-rectum	(-)(++)	(-)(++)↑ slight increase from stages I to IV	[19, 48]	(-) (++)↑	(-)(++)	(+)↓ decreas -ing from stage II to IV	(++) very slight increase from stages I to IV	[19, 20, 48]
Colon cancer cell lines (SW-48) (RKO)	 (++)↑ (++)↑	 (+)↑ (++)↑↑	 [48] [48]	 (++)↑ (++)↑	 (++) (++)	 (+) (+)	 (++)↑ (++)↑	 [48] [48]
Small intestine	(+)	(++)(-)	[56]					
Liver Cancer								

Cancer Type	Ghrelin			GHSR				
	mRNA	Protein	References	1a mRNA	1b mRNA	1a Protein	1b Protein	References
Liver cancer tissue				(++)				[20]
Hepatoma cell line (HepG2)				(++)				[25]
Lung Cancer								
Lung tumour	(++)↑		[26]	(++)↑	(++)↑			[26]
Lung endocrine tumours	(++)(+)	(++)	[28, 56]	(-)(++)↑				[27, 28]
Non-endocrine lung carcinomas	(++)	(-)	[28]	(-)				[27]
Non-small cell lung carcinoma (NSCLC)								
Adenocarcinoma							(++)↑	[29]
Squamous cell carcinoma							(++)↑	[29]
Small cell lung carcinoma (SCLC)	(++)		[56]				(++)↑	[29]
NSCLC cell lines (A549, NCIH358, NCI-H522, NCI-H1435, LC176, LC319, PC3, PC14)	(-)		[29]	(-)	(++)			[29]
(NCI-H23, NCI-H1793, LC174, PC9, SK-LU-1, RERF-LC-AI, SK-MES-1)	(-)		[29]	(-)	(-)			[29]
H345 Small cell lung carcinoma cell line	(++)		[28]	(-)				[28]
Ovarian Cancer								
Benign Tumours								
Serous cystadenoma		(++)	[380]			(+++)		[45]
Mucinous cystadenoma						(+++)		[45]
Brenner tumour						(-)		[45]
Malignant Tumours								
Serous cystadeno carcinoma, low grade		(++)	[380]			(+)		[45]
Serous cystadeno Ca, high grade		(+++)↑	[380]			(-)		[45]
Mucinous cystadeno carcinoma						(-)		[45]
Endometrioid cancer		(+)(-)↓	[46]			(-)		[45]
Clear cell carcinoma						(-)		[45]
Pancreatic Cancer								
Glucagonoma	(++)	(++)	[30, 31]	(-)				[31]
Insulinoma	(-)(++)	(-)	[30, 31]	(+++)				[31]
Non-Functioning	(++)	(++)	[31]	(+)		(++)		[31]
Gastrinoma	(++)		[31]	(+)				[31]
Well-differentiated neuroendocrine tumour	(+++) (++)	(++)	[56]					
Pancreatic adenocarcinomas cell lines								
(MIAPaCa2, BxPc3, Capan2)	(-)	(-)	[54]	(++)	(++)	(++)	(++)	[54]
(PANC1)	(+)	(-)	[54]	(++)	(++)	(++)	(++)	[54]
Pituitary Adenomas								
Somatotroph	I and II (+++) III and IV (+)	(++)	[32, 33, 37]	(+++)↑	(+++)↑			[32-36]
Corticotroph	(+)↓		[32, 33]	(++)	(++)			[32-35]
Gonadotroph	(++)		[32, 33]	(+)(++)	(+)			[32, 33, 35]
Non-functioning pituitary adenomas	(++) (+++)	(++)	[32, 33, 37]	(++)	(++)			[32, 33]
Lactotroph	(++)	(++)	[32, 33, 37]	(++)	(++)			[32-34, 36]
Thyrotroph	(++)		[33]	(++)↑				[33, 35]
Prostate Cancer								
Prostatic carcinomas	(++)	(-)(++)	[38, 40]	(-)	(-)			[38]
Benign hyperplasias	(++)	(-)	[38]	(-)	(++)			[38]
Androgen-independent cell lines (PC3, DU-145)	(++)	(++)	[38-40]	(++)	(++)↑	(++)		[38, 39]
Androgen-dependent cell lines		(++)	[39, 40]	(++)	(++)↑	(++)		[39]

Cancer Type	Ghrelin			GHSR				
	mRNA	Protein	References	1a mRNA	1b mRNA	1a Protein	1b Protein	References
(LNCaP, ALVA-41)								
Renal Cancer								
Clear cell renal carcinomas		(++)↓	[41]					
Chromophobe type renal cell carcinomas		(++)↓	[41]					
Papillary type renal cell carcinomas		(++)↓	[41]					
Oncocytoma		(++)↓	[41]					
Salivary Glands								
Mucoepidermoid carcinoma		(-)↓	[22]					
Testicular Cancer								
Leydig Cell Tumours								
Differentiated		(++)	[42]			(++)		[42]
Poorly differentiated		(-)↓	[42]			(++)		[42]
Germ Cell Tumours								
Seminoma		(-)	[42]			(++)		[42]
Embryonal carcinoma		(-)	[42]			(++)		[42]
Thyroid Cancer								
Medullary	(++)	(++)↑	[43, 56]					
Follicular	(+)	(++)↑	[44, 56]	(-)				[44]
Papillary	(+)		[56]					
Poorly differentiated carcinoma	(+)		[56]					
Medullary thyroid carcinoma cell line (TT)	(++)	(++)	[43]					
Parathyroid	(+)		[56]					

observed in carriers of the ghrelin gene SNPs rs171407-G and rs572169 [389]. This was correlated with a significant increase in height and IGF-I-circulating levels and interestingly, with rs171407-G, a decrease in BMI [389]. This contrasts with the association that has frequently linked increased cancer risk to increased BMI. This study also suggested that for the SNP, rs2948694, homozygotes had a 2-fold increase in the risk of breast cancer development [389]. Larger studies would be required to confirm this result, however [389]. Other SNPs in the ghrelin gene, rs27647 and rs35683, were associated with lower risk of colorectal cancer [388]. The *GHRL* SNP, rs27647, potentially shows a borderline protective effect against colorectal cancer, however, this requires further confirmation [388]. Another study in breast cancer also showed that some rare polymorphisms in the ghrelin gene (haplotypes GGAC and GGAT of SNPs: G-1062C, Arg51Gln, Leu72Met and 30-UTR T+673C where bases are cited in the order and variant alleles are underlined) had a possible protective influence on breast cancer risk [390].

Hypermethylation of the *GHSR* gene is seen in cases of infiltrating ductal breast cancer and can be used to successfully differentiate between breast cancer and benign tissue at a specificity and sensitivity higher than any other biomarker currently described [391]. Interestingly this differential methylation led to downstream downregulation of GHSR1a mRNA [391], which may lead to increased tumourigenesis. This provides further evidence that GHSR may play a role in cancer.

1.3.7.3 Ghrelin and processes related to cancer progression

Ghrelin has been shown to have effects on a number of the hallmarks of cancer, including cell proliferation, migration, invasion, apoptosis and angiogenesis (Table 1.4) [392, 393]. Ghrelin may, therefore, play a role in regulating cancer progression.

1.3.7.3.1 Ghrelin and cell proliferation and differentiation

Ghrelin is widely expressed and has an effect on a number of cellular functions that are key processes in cancer progression, including cell proliferation, migration and apoptosis. The role of ghrelin in cell proliferation has been widely studied, however, it appears to have different effects on some cell types and tissues of origin. Ghrelin increases proliferation in many normal cell types and cell lines, including skeletal muscle [394], placental [47], ovarian [395-398], pancreatic [399], brain [400],

preadipocyte [401-404], mesenchymal stem cell [405], bone [168, 189, 319], aortic [169], heart [172, 406], intestinal [407], adrenocortical [408, 409], microvascular endothelial [174], breast [17], pancreatic [54, 175], endometrial [49] and pituitary [57] cells or cell lines. Ghrelin decreased the rate of cell proliferation in some studies, in cell types including vascular smooth muscle [410, 411], aortic smooth muscle [285], lung [28], adrenal [13] and (testicular) Leydig [412] cells. While it has predominantly been demonstrated that treatment of normal cell lines with ghrelin increases cell proliferation [189, 320], this is not always the case in cancer cell lines. For example in thyroid cells, enhanced proliferation was seen in normal cells [413], while alternatively, in cancer an inhibition was observed [44].

Ghrelin has been shown to increase proliferation in a number of cancer and tumour cell lines (Table 1.4), including breast cancer [17], choriocarcinoma [47], colon cancer [48], endometrial cancer [49], erythroleukaemia [50], hepatoma [25], leukaemia [51], pancreatic adenocarcinoma [54], pancreatic neuroendocrine tumour [56], pituitary tumour [57], prostate cancer [38-40] and thyroid carcinoma [56]. Ghrelin has been demonstrated to decrease proliferation in adrenocortical carcinoma [53], thyroid carcinoma [44, 56] and prostate cancer [38]. Some of these differences may represent cell-type specific responses. In some breast and prostate cancer cell lines there is conflicting data, however [38-40, 414]. This may be the result of differences in the treatments, such as the concentration or purity of the peptide used [38-40], or due to differences in GHSR expression, even within the same cell line (Table 1.4). In the LNCaP and PC3 prostate cancer cell lines, an increase in cell proliferation has been demonstrated in some studies [39, 40] and a decrease in proliferation was described in the DU-145 and PC3 prostate cancer cell lines in other studies [38, 414]. In the TT thyroid cancer cell line the two forms of ghrelin had opposing effects on cell proliferation [56]. Acylated ghrelin caused a decrease in cell proliferation, while desacyl ghrelin led to an increase in proliferation [56]. Interestingly, both acylated and desacyl ghrelin induced an anti-proliferative effect *in vitro* in H345 small cell lung carcinoma cells and a dose-dependent pro-apoptotic effect was also seen, despite the absence of GHSR1a expression [28].

In a number of studies, ghrelin has been demonstrated to stimulate cell proliferation through the ERK1/2 MAPK signalling pathway [25, 40, 57, 168-172]. Ghrelin has also been shown to regulate proliferation through cAMP/PKA [169], NO/cGMP

Table 1.4 Summary of the effects of ghrelin on cell proliferation, apoptosis and cell migration in tumour cell lines. For each cancer type studied, the assay conditions (concentration of ghrelin used, use of inhibitors), the results of signalling studies linked to function, and presence of receptor expression and whether it is up or downregulated is given. Cell lines listed in bold indicate cell lines where conflicting data has been reported. * = statistically significant changes were observed with the treatment concentration compared to other concentrations tested; ↑ = increased expression/ phosphorylation; ↓ = decreased receptor expression/ or decreased phosphorylation

Function	Cancer cell line or cell type	Assay Conditions	Signalling	Receptor expression	Reference
Apoptosis					
Inhibition of apoptosis	Choriocarcinoma cell line – JEG-3	100, 250*, 500* or 1000 pg/ml	↓caspase-3		[47]
	Colon cancer cells – HT-29	10 nM*, 100 nM*, 1000 nM*	↓caspase-3, ↓Bax, ↑Bcl-2		[52]
	Endometrial cancer cell lines – HEC1B, Ishikawa, KLE	0.1 nM*, 1 nM*, 10 nM* octanoylated ghrelin		GHSR1a, GHSR1b	[49]
Increased apoptosis	Prostate cancer cell line – PC3	50 nM		GHSR1a, GHSR1b	[414]
No effect	Prostate cancer cell lines – LNCaP	0.1 nM to 1000 nM octanoylated ghrelin	↑ERK1/2, ↑p38 kinase		[40]
Invasion					
Increased	Astrocytoma cell lines – U-118, U-87, CCFSTTG1, SW1008	1 nM, 10 nM and 100 nM* ghrelin Inhibition with siRNA to GHSR1a	↑Ca^{2+} mobilisation, ↑PKC	↑GHSR1a	[16]
	Colon cancer cell lines – SW-48, ROK	Inhibition with GHS-R antagonist D-[lys-3]-GHRP-6 and ghrelin antibody		↑GHSR1a, GHSR1b mRNA GHSR1a, ↑GHSR1b protein	[48]
	Pancreatic adenocarcinomas cell line – PANC1, MIAPaCa2, BxPC3 and Capan2	10 nM* octanoylated ghrelin. Inhibition with GHS-R antagonist D-Lys-GHRP6	↑Akt	GHSR1a, GHSR1b	[54]
Migration					
Increased	Astrocytoma cell lines – U-118, U-87, CCFSTTG1, SW1008	1 nM, 10 nM and 100 nM* ghrelin Inhibition with siRNA to GHSR1a	↑Ca^{2+} mobilisation, ↑PKC	↑GHSR1a	[16]
	Colon cancer cell lines – SW-48, ROK	Inhibition with GHS-R antagonist D-[lys-3]-GHRP-6 and ghrelin antibody		↑GHSR1a, GHSR1b mRNA GHSR1a, ↑GHSR1b protein	[48]
	Glioma tumour cell lines – U251, C6 (rat)	Inhibition GHS-R antagonist and	↑CaMKII, ↑AMPK,		[55]

Function	Cancer cell line or cell type	Assay Conditions	Signalling	Receptor expression	Reference
		inhibitors to CaMKII, AMPK, NF-κB	↑NF-κB		
	Pancreatic adenocarcinoma cell line – PANC1, MIAPaCa2, BxPC3 and Capan2	10 nM* octanoylated ghrelin Inhibition with GHS-R antagonist D-Lys-GHRP6	↑Akt	GHSR1a, GHSR1b	[54]
Proliferation					
Increased	Breast cancer cell lines – MDA-MB-231, MDA-MB-435	0.1 nM* to 100 nM*, 1000 nM octanoylated ghrelin (note 0.1 nM and 1 nM significance MDA-MB-435 only)		GHSR1a, GHSR1b (note GHSR1a expression low in MDA-MB-231)	[17]
	Choriocarcinoma cell line – JEG-3	100, 250, 500 or 1000 pg/ml			[47]
	Colon cancer cell lines – SW-48, ROK	Inhibition with GHS-R antagonist D-[lys-3]-GHRP-6 and ghrelin antibody		↑GHSR1a, GHSR1b mRNA GHSR1a, ↑GHSR1b protein	[48]
	Endometrial cancer cell lines – HEC1B, Ishikawa, KLE	0.1 nM, 1 nM* to 100 nM*, 1000 nM octanoylated ghrelin		GHSR1a, GHSR1b	[49]
	Erythroleukaemia cell line – **HEL**	Inhibition with SB801* and SB969* ghrelin antisera		↑GHSR1b, no GHSR1a	[50]
	Hepatoma cell line – HepG2	100 nM*	↑MAPK, ↑IRS-1, ↑GRB2, ↑PI3K, ↓Akt	GHSR	[25]
	Leukemic cell lines – **HL-60** and **THP-1**	Inhibition with SB801* ghrelin antisera		GHSR1b, no GHSR1a	[51]
	Pancreatic adenocarcinomas cell line – PANC1, MIAPaCa2, BxPC3 and Capan2	1 nM, 10 nM* and 100 nM octanoylated ghrelin Inhibition with GHS-R antagonist D-Lys-GHRP6 caused a significant decrease with 100 nM	↑Akt	GHSR1a, GHSR1b	[54]
	Pancreatic neuroendocrine tumour cell line – BON-1	1 nM, 10 nM, 100 nM* octanoylated ghrelin			[56]
	Pituitary tumour cell line – GH3	0.001 nM, 0.1 nM* to 1000 nM* octanoylated and des-octanoylated ghrelin	↑pERK1/2 U0126 (ERK1/2 inhibitor), GF109203X (PKC inhibitor) and tyrphostin 23 (tyrosine kinase inhibitor) inhibited cell proliferation	GHSR1a	[57]

Function	Cancer cell line or cell type	Assay Conditions	Signalling	Receptor expression	Reference
	Prostate cancer cell lines – **LNCaP**, **PC3**	0.01 nM* to 10 nM*, 100 nM to 10000 nM octanoylated and des-octanoylated ghrelin	↑ERK1/2, ↑p38 kinase ERK inhibitors U0126 and PD98059 reversed proliferation effects	**LNCaP**; No GHSR1a or GHSR1b **PC3**; no/yes GHSR1a, no/yes GHSR1b	[38-40]
	Thyroid carcinoma cell line – TT	1 nM, 10 nM*, 100 nM* des-octanoylated ghrelin			[56]
Decreased	Adrenocortical carcinoma cell line – NCI-H295, SW13	0.1 nM*, 1 nM*, 10 nM*, 100 nM* 1000 nM*		↑GHSR1b, ↓GHSR1a	[53]
	Thyroid carcinoma cell lines – N-PAP, ARO, TT	10 nM, 100 nM*, 1000 nM* TT- 1 nM, 10 nM*, 100 nM octanoylated ghrelin		No GHSR1a or GHSR1b Alternative ghrelin receptor binding sites present	[44, 56]
	Prostate cancer cell line – DU-145,	0.01 nM, 0.1 nM, 1 nM* to 10000 nM* octanoylated and des-octanoylated ghrelin		GHSR1a, GHSR1b	[38]
	Prostate cancer cell line – **PC3**	1 nM* treatment only in one of two assay		No GHSR1a or GHSR1b	[38]
		1 nM, 3 nM, 10 nM*, 30 nM*, 50 nM*		GHSR1a, GHSR1b	[414]
No change	Breast cancer cell lines – MCF-10A, MCF7	0.1 nM to 1000 nM octanoylated ghrelin)		Low GHSR1a, GHSR1b (note only demonstrated in MCF7 cell line)	[17]
	Erythroleukaemia cell line – **HEL**	1 μm octanoylated ghrelin or des-octanoylated ghrelin		↑GHSR1b, no GHSR1a	[50]
	Gastric cancer cell line – KATO-III	500 nM			[11]
	Leukemic cell line – **HL-60**, **THP-1**, and SupT1	1 nM, 10 nM, 100 nM, and 1 μm octanoylated ghrelin or desacyl ghrelin		GHSR1b, no GHSR1a	[51]
	Lung carcinoma cell line – CALU-1	1 nM to 1000 nM		No GHSR1a	[27]
	Prostate cancer cell line - **LNCaP**	0.01 nM to 10000 nM octanoylated and des-octanoylated ghrelin		No GHSR1a or GHSR1b	[38]

[189], IRS-1 [25], GRB2 [25] and p38 [40] pathways.

Ghrelin may also promote differentiation in some cell types. *In vitro,* ghrelin promoted C2C12 skeletal myoblasts to differentiate and to fuse into multinucleated myotubes [394]. Ghrelin also stimulated differentiation of the osteoblastic cell lines, MC3T3-E1, ROS 17/2.8, UMR-106, MG63, and SaOS2 cells [319] and 3T3-L1 adipocytes/preadipocytes [401, 404].

Another important hallmark of cancer is the evasion of apoptosis (programmed cell death), allowing the propagation of damaged cells and a decrease in the rate of cell attrition [393]. In many studies, ghrelin has been demonstrated to inhibit apoptosis in cancer and normal cells and cell lines [47, 175-178, 181, 188, 395, 397-399, 410, 415-421] and to promote cell survival [175, 177, 415]. Additionally, the inhibition of apoptosis by ghrelin is often accompanied by increased proliferation [47, 319, 395, 397, 398], but it has also been associated with the inhibition of proliferation [411]. In contrast to most studies, a study in H345 small cell lung carcinoma cell line described an increase in apoptosis with ghrelin treatment, accompanied by the inhibition of proliferation [28]. Although in most studies in normal cell lines ghrelin inhibits apoptosis, this may differ with cell type, and a wide range of different stimuli have been used to elicit apoptosis. Ghrelin also has an anti-apoptotic effect in choriocarcinoma [47], colon cancer [52] and endometrial cancer [49] cell lines. A single study has indicated that ghrelin promotes apoptosis in the PC3 prostate cancer cell line [414]. The majority of studies suggest that ghrelin promotes cancer progression.

Desacyl ghrelin appears to have mostly similar effects to ghrelin on cell proliferation and apoptosis. Desacyl ghrelin, like ghrelin, increases proliferation in a number of normal cell types including bone [168], HIT-T15 and INS-1E pancreatic beta-cell lines [175] and the 3T3-L1 adipocyte cell line [422] and inhibits apoptosis in cardiomyocyte cells and the H9c2 cell line [178], endothelial cells [178] and pancreatic cells and cell lines [175, 177, 415]. Both desacyl ghrelin and acylated ghrelin were shown to promote cell survival in pancreatic β-cell lines and human pancreatic islets [175, 415] and in cardiomyocytes and endothelial cells [178]. Both major forms of ghrelin protect cortical neurons against ischaemic injury [421] and

glucose deprivation-induced apoptosis [180]. Desacyl ghrelin inhibits proliferation and increases apoptosis in the H345 small cell lung carcinoma cell line in a dose-dependent manner [28].

Ghrelin inhibits apoptosis by signalling through the ERK1/2 MAPK signalling pathway [175-183]. Protection against apoptosis has also been shown to be mediated by cAMP/ protein kinase A (PKA) [177], TNF-α/NF-κB [187], protein kinase C (PKC) [188], caspase-3 [47, 52, 180], Bax [52, 180], Bcl-2 [52, 180] and glycogen synthase kinase-3beta (GSK-3β) [180]. Inhibiting adenylyl cyclase/cAMP/PKA signalling prevented the ghrelin cytoprotective effect against apoptosis induced by serum starvation or interferon (IFN)-γ /TNF-α in HIT-T15 β -cells [175].

1.3.7.3.2 Ghrelin and cell migration and invasion

Ghrelin stimulates cell migration in normal derived cell lines, including human microvascular endothelial cells (HMVEC) [174], cardiac microvascular endothelial cells (CMECs) [171], and in cancer cell lines including U251 and C6 glioma cell lines[55], CCFSTTG1, U118, U87 and SW1008 astrocytoma cell lines [16], PANC1, MIAPaCa2, BxPC3 and Capan2 pancreatic adenocarcinoma cell lines [54], and SW-48 and RKO colon cancer cell lines [48]. These studies showed that the increased migration could be inhibited by antagonising the ghrelin receptor [48, 54, 55, 190]. The effect of ghrelin on promoting migration in the colon cancer cell lines was also demonstrated by blocking ghrelin using an antibody [48]. Migration is a complex process involving the extension of a protrusion, through the polymerisation of actin filaments, from the leading edge of the cell membrane, adhesion of this protrusion and retraction of the rest of the cell in the direction of motility [423-425]. Studies have shown that ghrelin may influence the remodelling of the cytoskeleton needed for migration. In human T cells, human astrocytoma cell lines and rat hippocampal slice cultures, ghrelin stimulates actin polymerisation [16, 267, 426]. In astrocytoma motility studies, using scratch and Transwell assays, ghrelin stimulated increased migration with increased actin polymerisation [16]. Increases in intracellular calcium mobilisation, PKC activation, MMP2 activity and co-localization of the small GTPase, Rac1, with GHSR on the leading edge of the cells and membrane ruffling on cells was also observed [16]. In the astrocytoma cell lines, the increased migration was shown to be dependent on GHSR1a by disrupting the endogenous ghrelin-GHSR

pathway by siRNA [16].

Ghrelin also appears to stimulate cell invasion in the PANC1, MIAPaCa2, BxPC3 and Capan2 pancreatic adenocarcinoma cell lines [54]. Inhibition of ghrelin receptor signalling with the GHSR inverse agonist D-[lys-3]-GHRP-6 reduced cell invasion in SW-48 and RKO colon cancer cell lines [48] and pancreatic adenocarcinomas cell lines [54].

Ghrelin-stimulated migration and invasion is mediated through a variety of signalling pathways. In glioma cell lines, rat C6 and human U251, ghrelin-stimulated cell migration was prevented by inhibition of GHSR using an antagonist, and through inhibition of the CaMKII, AMPK and NF-κB signalling pathways, indicating a role for these signalling pathways [55]. Inhibition of the cAMP/PKA pathway and AMP cyclase has also been demonstrated to knockdown ghrelin-stimulated migration in human aortic endothelial cells [190, 285]. Ghrelin also stimulates cell migration and invasion by stimulating the ERK1/2 MAPK pathway [172, 174], and in astrocytoma, ghrelin signalling stimulates cell migration by signalling through PKC [16].

1.3.7.3.3 Ghrelin and angiogenesis

Ghrelin plays a role in regulating angiogenesis, and we hypothesise that ghrelin may also play a role in angiogenesis in cancer progression. Angiogenesis currently represents a major therapeutic target for cancer, because of the potential to control the growth of tumours which are dependent upon abundant vascularisation [427], and synthetic ghrelin analogues (agonists) could be useful anti-angiogenic therapeutics [428]. Ghrelin is expressed in blood vessels [275, 429], however, its ability to influence angiogenesis has not been widely explored. Ghrelin and GHSR are expressed in human umbilical vein endothelial cells (HUVECs), in human microvascular endothelial cells (HMVEC) [174] and in cardiac microvascular endothelial cells (CMECs) [171, 172]. In HUVECs, ghrelin inhibits FGF-2-mediated angiogenesis [428]. Ghrelin also stimulates antiangiogenic action in rat brain microvascular endothelial cells [191]. In rat CMECs and in HMVECs, ghrelin significantly increased angiogenesis, cell proliferation and migration [171, 172, 174]. In a study comparing neonatal (neo) HMVECs and HMVECs derived from aging individuals (66 years and 90 years old) who were known to have impaired

angiogenesis, ghrelin levels were reduced in the aged group compared to the neo HMVECs [430]. Treatment with exogenous ghrelin significantly reversed impaired angiogenesis displayed by the aged HMVEC line. [430].These studies found that ghrelin-stimulated angiogenesis is mediated through the ERK1/2 MAPK pathway [171, 172, 174, 430], whereas a previous study suggested that ghrelin was causing an inhibition of MAPK pathway [191]. Ghrelin-stimulated angiogenesis has also been shown to be mediated by decreasing tyrosine kinase (TK) activity [191].

1.4 Obestatin

1.4.1 Obestatin peptide

Processing of the preproghrelin peptide gives rise to at least two different peptide hormones, ghrelin [1] and obestatin. Obestatin is a highly conserved, 23 amino acid peptide hormone [6] and it is cleaved from preproghrelin and the C-terminal, C-ghrelin peptide [6]. The C-ghrelin peptide is derived from the cleavage of the ghrelin peptide from preproghrelin by prohormone convertases PC1/3, PC2 and furin (Fig. 1.2) [122]. The coding region for obestatin begins at the start of exon 3 of the preproghrelin transcript [6]. The region encoding obestatin is flanked by potential convertase cleavage sites, and at its C-terminus, it has conserved glycine residue suggesting a requirement for amidation [431]. Currently, little is known about the processing of preproghrelin into obestatin, or its regulation. It is predicted that obestatin is derived by enzymatic cleavage from the full-length preproghrelin transcript [432], however, this mechanism has yet to be demonstrated. Obestatin is expressed in the same cells in the stomach as ghrelin [433] and circulates in the plasma as a hormone, but is rapidly degraded [434]. Unlike acylated ghrelin, it is unable to cross the blood-brain barrier [136, 435].

Like ghrelin, the stomach is the primary site of obestatin expression [6, 433, 436, 437]. It is also expressed in a range of other peripheral tissues, including the pancreas [12, 56, 433, 436, 437], pituitary [56], gastrointestinal tract [56, 433, 436], lung [56], thyroid [56, 438], liver [437], testis [439] and mammary gland [436, 440]. Obestatin is present in seminal fluid and has been positively correlated with sperm concentration and motility [441]. Obestatin circulates at a much lower concentration in plasma than ghrelin [442]. The plasma levels of obestatin are correlated to body

weight, gender and food intake, but not to age [443, 444]. Plasma levels of obestatin in obese women are lower than normal weight and anorectic women [445]. The ghrelin to obestatin ratio is increased in anorectic patients. [445, 446] and a number of studies have demonstrated that the ghrelin to obestatin ratio changes in disease states, such as in obesity, anorexia nervosa and hypertension [445, 447]. Interestingly, C-ghrelin, the peptide from which obestatin is derived, circulates in the plasma at higher levels than ghrelin [448, 449]. Although the reason for this is unclear, this may indicate that the peptides are differentially regulated, or it may be due to differences in their half-lives [448, 449]. It is also possible that novel obestatin-specific transcripts are also translated and these could influence the circulating ghrelin to obestatin ratio [118].

1.4.2 The obestatin receptor

The obestatin receptor was originally thought to be the orphan receptor, GPR39 [6, 450], a member of the small ghrelin receptor subfamily [159, 451]. Other research groups have failed to reproduce the initial findings and it is now widely accepted that GPR39 is not the obestatin receptor [452-455]. GPR39 is a G protein-coupled receptor, expressed in the brain and surrounding tissues [451, 456]. GPR39 mRNA is expressed in multiple tissues, including the stomach, adipose tissue, intestine, endocrine pancreas, liver and hypothalamus, and this is consistent with a role in energy balance [6, 451, 457-459]. It is now emerging the GPR39 may act as an important transducer of autocrine and paracrine Zn^{2+} signals, influencing cellular processes such as insulin secretion, gastric emptying, neurotransmission and epithelial repair [460]. It was hypothesised that the glucagon-like peptide 1 receptor (GLP-1R) could be an obestatin receptor [12], however, a subsequent study refuted this finding, showing that obestatin did not bind GLP-1R, nor displace the GLP-1R ligand [461]. Binding sites for obestatin have been identified in the pancreas, heart and white adipose tissue [12, 450, 462, 463], however, the identity of the obestatin receptor remains elusive.

Although the identity of the obestatin receptor is unknown, some studies have investigated obestatin signalling pathways. Obestatin appears to activate an unknown GPCR [10, 11, 439, 462, 464-466]. There is also evidence that obestatin may stimulate receptors that are expressed by the vagus and signal to the brain through

vagal afferent pathways [467, 468], like ghrelin, to regulate gastrointestinal motility [469-471]. It also appears that, like ghrelin, obestatin stimulates the ERK1/2 signalling pathway in mediating functions such as cell proliferation, the inhibition of apoptosis and in stimulating growth hormone secretion [10-12, 462, 465, 466]. Obestatin has also been shown to activate the Akt signalling pathway [464, 472] and inactivate AMPK [464]. Induction of Akt signalling has been linked to the stimulation of proliferation [473]. In 3T3-L1 adipocyte cells, obestatin activates Akt and its downstream targets, glycogen synthase kinase 3α/β (GSK3α/β), mTOR and S6 kinase 1 (S6K1), and Akt signalling plays a role in obestatin-stimulated adipocyte metabolism and adipogenesis [464]. This is accompanied by the simultaneous inactivation of AMPK and a decrease in Acetyl-CoA carboxylase (ACC) phosphorylation [464].

1.4.3 The function of obestatin

Obestatin, which is produced from the same prepropeptide as ghrelin, was originally described as opposing the effect of ghrelin in appetite and bodyweight [6]. This appetite-suppressing function inspired the name, obestatin, which is derived from the Latin contraction for obese, *obedere*, meaning to devour, and *statin*, denoting suppression [6]. There has been much controversy, however, over the role of obestatin, as the effects on appetite and energy balance have proven difficult to repeat in other laboratories [474-479]. Some researchers have proposed that obestatin should be renamed *ghrelin associated peptide,* as it may not suppress appetite [476]. It has now been shown to have functions unrelated to the regulation of appetite [7].

1.4.3.1 The effect of obestatin on feeding, gut motility, energy balance and thirst

Obestatin was originally demonstrated to oppose the effects of ghrelin, suppressing food intake, gut motility and weight gain in mice through GPR39 [6, 136]. This was opposite to the effect seen in mice in response to acylated ghrelin [1]. As indicated above, these results have not been readily reproducible. The anorexigenic effects of obestatin in fasted/refed mice and decreased gastrointestinal motility have not been reproduced by other groups [435, 439, 453, 474-476, 480-486], with the exception of two studies [9, 487]. In one of the studies in which decreased feeding was observed, obestatin was dissolved in dimethyl sulphoxide (DMSO)/saline immediately prior to intraperitoneal injection, while the control group received saline without DMSO

[487]. No other studies used DMSO to dissolve obestatin [6, 9, 435, 453, 474, 480-484] and this may have confounded the data [487]. DMSO is likely to have independent effects and it is not recommended as a vehicle in feeding studies [488]. The negative findings obtained by other groups cannot be attributed to differences in experimental design, and a number of studies have mimicked the original test conditions [435, 482, 483], while others have been performed under comparable conditions [453, 474, 480, 481, 484]. The use of comparative control peptides, including ghrelin, revealed the expected food intake modifications under the same conditions [453, 474, 480-484]. Additionally, when obestatin was co-administered with ghrelin in fasted/refed mice or rats, as in the paper by Zhang *et al.*, suppression of ghrelin's effect was not seen [435, 474, 484]. Despite these results, a ghrelin-opposing effect of obestatin was observed in one study when obestatin and ghrelin were injected peripherally at 1μmol kg^{-1} during the light-off period in freely fed mice [435]. Obestatin circulates at high levels in the serum (0.3ng/mL), however, unlike ghrelin, these level were unaffected by feeding and fasting, suggesting that its expression is not regulated by feeding [6, 136].

The initial finding that obestatin decreased gastrointestinal motility [6] has not been successfully reproduced in a number of *in vivo* or *in vitro* studies [439, 477, 483, 485, 486]. The original study was performed in mice, however, while the subsequent gastrointestinal motility studies were performed on rats, except one which used both mice and rats [485]. Obestatin was found by one group to exert inhibitory effects on the motility of antrum of the stomach and the duodenum in fed rats [469-471].

Despite controversies regarding its effect on feeding, obestatin may prevent thirst. Obestatin inhibited thirst in a number of rat studies [480, 489], in *ad libitum*-watered and angiotensin II - and hypovolemia- induced thirst [480, 489]. This was not validated by another independent study where the effect of obestatin administration on *ad libitum*-watered mice over a period of time showed no effect on water intake [490]. It has been suggested that effects of obestatin on feeding may be secondary to the inhibition of thirst as part of a phenomenon termed dehydration anorexia [480].

1.4.3.2 Obestatin and adiposity

Obestatin may play a role in regulating bodyweight by influencing adiposity.

Obestatin expression is increased during adipogenesis, and it promotes adipogenesis in an autocrine/paracrine manner by regulating adipocyte metabolism [7]. It has also been reported to have adipogenic effects in the 3T3-L1 adipocyte cell line [464]. Obestatin can induce AS160 phosphorylation, and translocation of the glucose transporter, GLUT4, to the plasma membrane, which allows insulin-regulated glucose transport into the cell, and augmented glucose uptake in 3T3-L1 adipocytes [464]. Neutralisation of endogenous obestatin using an anti-obestatin antibody decreases the rate of adipocyte differentiation [464]. In a recent study of mice fed a high fat diet obestatin prevented apoptosis of 3T3-L1 preadipocytes by stimulating PI3K/Akt and ERK1/2 signalling [491]. In the same study, obestatin also inhibited isoproterenol-induced lipolysis, promoted AMPK phosphorylation, and induced adiponectin, and reduced leptin secretion in both mouse (3T3-L1) and human (human subcutaneous and omental) adipocytes [491]. Peripheral injection of obestatin induces expression of the early response gene, *c-fos*, in gastric mucosa, intestinal villi, white adipose tissue, hepatic cords, and kidney tubules [450] and leads to decreased expression levels of the cholesterol transporter ABCA1 (ATP-binding cassette A1) in adipose tissue, suggesting changes in cholesterol transport [492].

1.4.3.3 Obestatin and growth hormone (GH) release

While ghrelin stimulates the release of growth hormone (GH) from the anterior pituitary, the majority of studies indicate that obestatin does not influence GH release [6, 480, 482, 484, 487]. Nevertheless, one study in rat somatotroph tumour cells has reported that obestatin stimulated GH release within 30 min and this had dissipated by one hour [466]. These authors suggested that obestatin stimulates GH release through the ERK1/2 signalling pathway [466]. Other studies suggest that obestatin is only effective in inhibiting ghrelin-stimulated responses. Obestatin inhibited ghrelin-stimulated excitation of growth hormone releasing hormone (GHRH) neurons and stimulated GHRH release through a receptor interaction which was independent of GHSR [493]. Obestatin has also been shown to inhibit ghrelin-stimulated GH release itself [435]. It is, therefore, currently unclear whether or not obestatin has a role in regulating GH release.

1.4.3.4 Obestatin and pancreatic function

Obestatin is co-expressed with ghrelin in the β-cells of the endocrine pancreas and the pancreatic islets [12, 56, 124, 436, 494, 495]. Pancreatic β-cells and human islets also express obestatin and specific obestatin binding sites [12]. Obestatin appears to play a significant role in the endocrine pancreas, working as an autocrine/paracrine factor, promoting β-cell growth and survival, allowing maintenance of islet size, and protecting against diabetes [12, 495].

The effects of obestatin on insulin release remain controversial, and reports have been conflicting. Studies have demonstrated that obestatin stimulates insulin secretion, inhibits secretion, or has no effect [12, 496, 497]. In human HIT-T15 β-cell line assays, obestatin increases the secretion of insulin and regulates GLP-1R, insulin receptor substrate-2 (IRS-2), and pancreatic and duodenal homeobox-1 genes [12]. Studies in rat and mouse models have mostly shown that obestatin inhibits insulin release [498-500]. In a study in rats, 10 nM obestatin inhibited insulin release, and low concentrations of obestatin (1 nM) stimulated insulin secretion [497]. Further studies are required to determine whether the effects of obestatin are dose dependent, or whether obestatin has different effects in human compared to rats and mice. A recent study demonstrated that obestatin enhanced glucose uptake in the presence and absence of insulin, promoted GLUT4 translocation, and increased Akt phosphorylation and sirtuin 1 protein (increases insulin sensitivity) expression in mice fed a high fat diet [491]. This enhanced glucose uptake could be reversed by the inhibition of sirtuin 1 by small interfering RNA [491]. Obestatin has also been demonstrated to stimulate the exocrine pancreas and the production of pancreatic juice, which is essential for efficient digestion [467, 468].

Obestatin inhibits apoptosis and promotes survival in the HIT-T15 and INS-1E pancreatic β-cell lines by increasing cAMP and activating ERK1/2 and phosphatidylinositol 3-kinase (PI3K)/Akt [12]. It also exerts anti-apoptotic effects through the inhibition of adenylyl cyclase/cAMP/protein kinase A (PKA), PI3K/Akt, and ERK1/2 signalling [12].

1.4.3.5 Obestatin and cardiovascular functions

There is evidence that obestatin may also have cardiovascular effects. In an isolated

rat heart preparation, treatment with obestatin before the induction of ischemia reduced infarct size and contractile dysfunction in a concentration-dependent manner [462]. Additionally, it was demonstrated that obestatin reduced cardiomyocyte apoptosis and it reduced caspase-3 activation in the rat H9c2 cardiac cell line and in isolated ventricular myocytes [462]. Specific obestatin binding sites have been demonstrated predominantly in the membranes of the ventricular myocardium and cultured cardiomyocyte models of ischemia-reperfusion [462]. The cardioprotective effects of obestatin in rat H9c2 cardiac cells and isolated ventricular myocytes are also mediated by PI3K and PKC in addition to ERK1/2 [462].

1.4.3.6 Obestatin and sleep, memory and anxiety and thermogenesis

There have been few studies demonstrating a role for obestatin in higher order functions, including sleep, memory and mood. Obestatin may act as a sleep-promoting factor when centrally administered to rats [8]. The pre-treatment of preproghrelin knockout mice with obestatin attenuated hypothermic responses, causing loss of sleep during torpor in mice [501]. Like ghrelin, obestatin improved memory retention, however, it also opposed the effects of ghrelin, causing reduced anxiety while ghrelin caused increased anxiety [9].

1.4.3.7 Obestatin and reproduction

There is limited information describing a role for obestatin in reproduction. Obestatin has been detected in the rat stomach, pancreas and plasma in the perinatal period [316]. This concentration of obestatin in rats is abruptly reduced after birth, when levels of ghrelin increase [316]. The reduction in pancreatic obestatin is continued until weaning [316, 502]. Additionally, in pregnancy, obestatin has been associated with the regulation of maternal blood pressure during the third trimester [503]. Obestatin is also expressed in porcine ovaries, regulating granulosa cell function and secretion [465]. Obestatin increased the accumulation of proliferation markers cyclin B1, proliferating cell nuclear antigen (PCNA), MAPK and the apoptosis markers p53, Caspase 3 and Bax in granulosa cells [465]. Increased secretion of progesterone was also observed [465].

1.4.4 Obestatin in disease

Obestatin has a diverse range of functions, however, there have been few studies

indicating a role in disease. Obestatin exerts cardioprotective effects in ischemic-reperfused isolated rat heart [462]. A decreased obestatin/ghrelin ratio has also been linked to inflammatory bowel disease [504].

1.4.5 Obestatin in cancer

1.4.5.1 Expression of obestatin in cancer

Obestatin is expressed in a number of cancer tissues and cell types (Table 1.5) and may be differentially expressed in cancer tissues compared to normal tissue. In a study of patients who underwent surgery for benign ovarian tumours (22 patients) and ovarian cancer (31 patients) and control women (32 patients), peripheral blood obestatin concentrations were measured [114]. Obestatin levels were higher in the blood of patients with both benign ovarian tumours and ovarian cancer compared to normal women with no expression of the disease [114]. In patients with prostate cancer [384] and uterine leiomyoma [383] levels of obestatin were not elevated compared to normal controls. Further studies are required to determine if plasma obestatin levels may be elevated in some cancers and if obestatin may be useful as a biomarker.

1.4.5.2 Obestatin and processes related to cancer progression

The role of obestatin in cancer has been the subject of few studies and its role in cell migration, invasion, apoptosis and angiogenesis has not been investigated. Obestatin has been shown to have differential effects on proliferation in cancer compared to normal cell lines [11, 56, 466].

1.4.5.2.1 Obestatin and cell proliferation and apoptosis

Cancer progression is characterised by a number of changes in basic cellular function including self-sufficiency in growth signals, insensitivity to growth-inhibitory (anti-growth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential (proliferation), sustained angiogenesis, and tissue invasion and metastasis [393]. Obestatin may act as a growth factor and, like ghrelin, it can alter cell proliferation in a number of cell types [7]. Exogenous obestatin treatment stimulates cell proliferation in some normal cell types, including human retinal epithelial cells [10], pancreatic β-cells and human pancreatic islet cells [12]. In porcine ovarian

Table 1.5 Expression of obestatin mRNA and protein in tumour tissues and cell lines. (-) no expression, (++) is expressed or has moderate expression, (+) low expression, (+++) high expression, ↓ lower levels of expression than normal tissue.

Cancer Type	Obestatin		
	mRNA	Protein	References
Adrenal tumours			
Phaeochromocytoma	(+)	(-)	[56]
Adrenocortical adenoma	(+)	(-)	[56]
Adrenocortical carcinoma		(-)	[56]
Appendix			
Well-differentiated neuroendocrine tumour		(-)(++)	[56]
Gastrointestinal Tract cancer			
Oral squamous cell carcinoma		(+)(-)↓	[24]
Oesophagogastric Adenocarcinomas			
Stomach	(+++)	(++)	[56]
Colon-rectum		(-)	[56]
Small intestine	(+)	(-)(++)	[56]
Lung Cancer			
Lung endocrine tumours	(+)	(-)	[56]
Small cell lung carcinoma (SCLC)	(++)	(-)	[56]
Ovarian Cancer			
Benign serous tumours		(++)	[380]
Borderline serous tumours		(++)	[380]
Malignant serous tumours		(++)	[380]
Pancreatic Cancer			
Gastrinoma			
Well-differentiated neuroendocrine carcinoma		(++)	[56]
Well-differentiated neuroendocrine tumour	(+++) (++)	(-)(++)	[56]
Pituitary Adenomas			
Adenoma		(-)	[56]
Thyroid Cancer			
Medullary	(++)	(-)(++)	[56]
Follicular	(+)	(-)(++)	[56]
Papillary	(+)	(-)(++)	[56]
Poorly differentiated carcinoma	(+)	(-)(++)	[56]
Parathyroid	(+)	(-)(++)	[56]

granulosa cells obestatin stimulated expression of proliferation markers (PCNA, cyclin B1, and MAPK) and markers of apoptosis (Bax, p53, Caspase 3) [465]. Like ghrelin, obestatin has different effects on cell proliferation in different cell lines. Obestatin inhibits proliferation in rat adrenocortical cells in a model of bilateral enucleation-induced adrenocortical regeneration [13], in the human C28-I2 rib chondrocyte cell line and the ATDC5 mouse embryonic carcinoma-derived cell line (after they are induced to differentiate into chondrocytes) [14].

The effect of obestatin on cell proliferation varies with cell type. Obestatin enhances proliferation in the KATO-III gastric cancer cell line [11], but inhibits proliferation in the TT thyroid carcinoma cell line [56] (Table 1.6). Obestatin had no effect on cell proliferation in the BON-1 pancreatic neuroendocrine tumour cell line [56], or in the GC rat somatotroph tumour cell line [466] (Table 1.6).

Table 1.6 The effects of obestatin on cell proliferation in tumour cell lines.
Cancer cell type, the concentration of obestatin used in assays, the use of inhibitors and any signalling data linked to the function are listed. * = significant changes observed with that treatment concentration compared to other concentrations tested; ↑ = increased expression/phosphorylation.

Function	Cancer Type	Specifics	Reference
Proliferation			
Increased	Gastric cancer cell line – KATO-III	50 nM* to 1000 nM* ↑PKC, ↑ERK1/2 Inhibition with pertussis toxin, PD98059 (ERK inhibitor), wortmannin (PI3K inhibitor), staurosporine (PKC inhibitor), PP2 (Src inhibitor)	[11]
Decreased	Thyroid carcinoma cell line – TT	1 nM, 10 nM*, 100 nM*	[56]
No change	Pancreatic neuroendocrine tumour cell line – BON-1	1 nM, 10 nM, 100 nM	[56]
	Somatotroph rat tumor cell line – GC	100 nM, 200 nM, 500 nM ↑ERK1/2	[466]

In the KATO-III gastric cancer cell line, obestatin-induced proliferation was mediated by the ERK1/2 pathway [11]. Further analysis of the obestatin transmembrane signalling pathway revealed that PI3K, PKC and Src inhibitors blocked activation of ERK1/2, suggesting that they are upstream of ERK1/2 in the mediation of obestatin signalling [11]. In another study in the KATO-III and AGS gastric cancer cell lines, obestatin activated Akt signalling through two potential

pathways involving GPR39 [472], which is surprising as it is now thought that GPR39 is not the obestatin receptor. Obestatin was thought to induce an association between GPR39 and a β-arrestin 1/Src signalling complex, resulting in transactivation of the epidermal growth factor receptor (EGFR) [472]. This leads to downstream Akt signalling, which is recognised as a signalling pathway that mediates cell proliferation [473]. Activation of this pathway was paralleled by the phosphorylation of mTOR (mammalian target of rapamycin) which can lead to Akt phosphorylation, or it can be activated downstream of Akt [472]. In primary human retinal epithelial cells, obestatin stimulated cell proliferation through ERK1/2 signalling, and the activation of Gi, PI3K, PKC and then Src [10].

1.5 Ghrelin and obestatin in the ovary and in ovarian cancer

In this study we have investigated the role of ghrelin and obestatin in processes related to cancer progression in ovarian cancer. We have previously demonstrated that ghrelin mRNA is expressed in the IOSE normal ovarian cell line, the HS 832 benign ovarian cyst and PEO14, SKOV3, PEO1, JAM, TOV 112D, OV90, ES2 and OVCAR3 ovarian cancer cell lines and from ovarian cancer tissue (Jeffery, unpublished data) [505]. We have shown that GHSR1a is expressed in the OVCAR3 ovarian cancer cell line using immunohistochemistry and in ovarian cancer specimens using RT-PCR [505]. Using real time RT-PCR, we demonstrated that exon 3 deleted isoform of ghrelin is relatively over-expressed in the more aggressive PEO14, SKOV3, PEO1, and JAM ovarian cancer cell lines compared to the IOSE, HS 832, TOV 112D, OV90 and ES2 cell lines (Jeffery, unpublished data) (Fig. 1.7).

Ghrelin, obestatin and GHSR protein expression has been demonstrated in ovarian cancer. In a small recent immunohistochemical study of benign (n = 20), borderline (n = 7) and malignant (n = 20) serous tumours, ghrelin, but not obestatin immunoreactivity was increased in the malignant tissues compared to benign tissues [380]. GHSR1a protein expression has been demonstrated immunohistochemically in the ovarian surface epithelium and Műllerian duct derivatives, as well as in benign serous tumours resembling Fallopian tube epithelium and in highly differentiated serous cystadenocarcinomas [45]. GHSR1a was not expressed in other ovarian neoplasms in this study, including mucinous cystadenomas, cystadenocarcinomas, endometrioid tumours, clear cell carcinomas or Brenner's tumours [45].

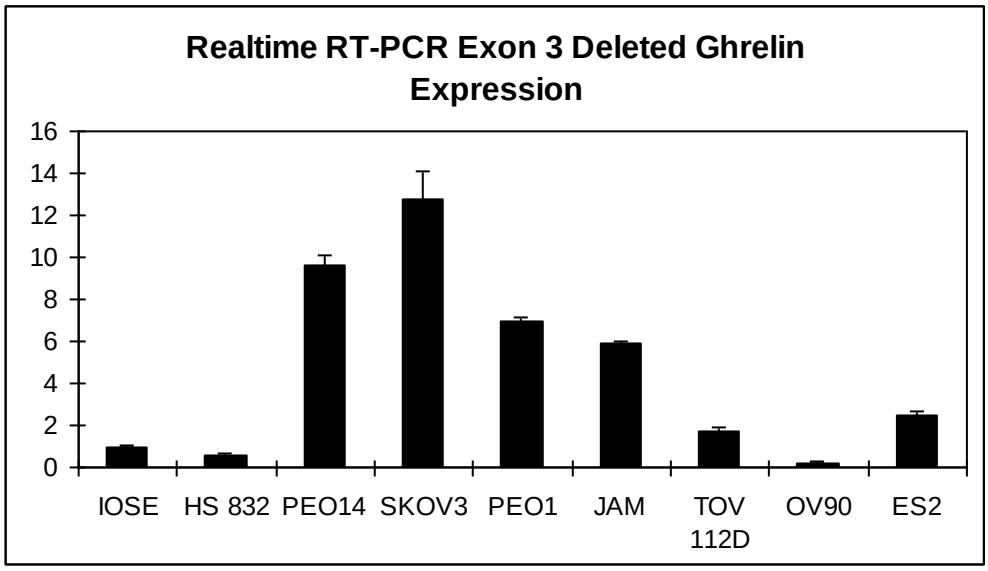

Figure 1.7 Real time RT-PCR expression of exon 3 deleted ghrelin in ovarian cancer cell lines. Data were normalised to 18s and are represented as fold changes, quantified as $2^{-(\Delta Ct\ sample-\Delta Ct\ control)}$, relative to expression of transcripts in IOSE normal ovarian cell line sample (1.0). Bars indicate mean +/- SEM. Unpublished data from Jeffery *et.al.*

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Our group and others, using immunostaining of histological tissue sections, have also shown that ghrelin and its receptor are expressed in the normal ovary, including ovarian follicles, and thecal cells within larger follicles, in larger oocytes, granulosa cells, hilus interstitial cells. They are expressed in the luteal compartment of the

ovary, and particularly in steroidogenic cells from corpus luteum (CL) of the current cycle and in the regressing CL [155, 287, 506]. In early development, however, ghrelin immunoreactivity was not detected in ovarian follicles at any stage, nor was it present in newly formed or regressing CL [155].

At the mRNA level in rat ovaries, ghrelin expression varied depending on the stage of the oestrous cycle [287]. During pregnancy, however, expression was detected persistently in the rat ovary, with higher levels of ghrelin in early pregnancy and lower expression during the later part of gestation [287]. In human luteal cells isolated from corpora lutea (collected from 23 normally menstruating patients in the midluteal phase of the menstrual cycle), and primary cultures, both desacyl ghrelin and obestatin treatment reduced progesterone and vascular endothelial growth factor (VEGF) release [507]. Obestatin was also able to reduce prostaglandin E2 (PGE2) and prostaglandin (PG) $F_{2\alpha}$ release [507]. Ghrelin is also a potent regulator of the secretory activity of chicken ovarian cells and of tyrosine kinase (TK), mitogen-activated protein kinase (MAPK) and protein kinase A (PKA) activity in the chicken ovary [291]. Furthermore, it has been suggested that MAPK-, cyclin-dependent protein kinase (CDK)- and PKA-dependent intracellular mechanisms are involved in the control of ovarian secretion, which may mediate the effects of ghrelin on these processes in the chicken [508]. In the porcine ovary ghrelin has been shown to stimulate cell proliferation and apoptosis mediated through the ERK1/2 and PI3 kinase pathways [509]. In another study in the porcine ovary, it was suggested that ghrelin increased proliferation, however, this study only measured an accumulation of proliferation markers (PCNA, cyclin B1, MAPK) [395]. Little is still known about the function of ghrelin and obestatin in the human ovary and in ovarian cancer.

1.6 Outline of project

Ovarian cancer is the leading cause of death among gynaecological cancers world wide. The incidence of ovarian cancer increased dramatically after the age of 50, with 81% of all new cases being diagnosed in this age group. With less than a 1% decline in the incidence of ovarian cancer over the past two decades and the mortality rate remaining largely unchanged, improved adjunctive therapies and markers for this disease are urgently required, as current methods for diagnosis and treatment are less than satisfactory.

There is increasing evidence for a functional role of the ghrelin axis (including ghrelin, obestatin and their receptors) in the progression of cancer [7, 15]. Cancer progression is characterised by a number of well defined steps named the hallmarks of cancer [392, 393] and ghrelin and obestatin have been demonstrated to influence a number of these hallmarks including cancer cell proliferation, migration (ghrelin only) and invasion (ghrelin only) [7, 15].

The effects ghrelin and obestatin on human ovarian cancer cell function remain unknown. In the porcine ovary, ghrelin has been shown to increase follicular and granulosa cell proliferation [395, 509]. Previous research in the Ghrelin research group has demonstrated that ghrelin mRNA is expressed in ovarian cancer cell lines and tissues [505]. Additionally, a recent immunhistochemical study has shown increased expression of ghrelin, but not obestatin, in malignant serous ovarian tumours compared to benign tissues [380]. In this thesis the effects of ghrelin and obestatin on ovarian cancer cell line functions, including cell proliferation, cell migration and invasion and cell attachment are investigated and compared to a normal ovarian cell line. This research may provide novel avenues for the development of novel biomarkers, or therapeutic strategies for ovarian cancer.

1.6.1 Hypotheses

We hypothesise that:

- components of the ghrelin axis are expressed in human ovarian cancer tissues.
- ghrelin and obestatin play a role in stimulating cell proliferation in ovarian cancer.
- ghrelin and obestatin may play a role in stimulating cancer-related processes including cell migration, invasion and cell attachment in ovarian cancer.

1.6.2 Specific Aims

I therefore aim to investigate:

(1) the cellular effects of exogenous obestatin and ghrelin in cell proliferation, migration, invasion and attachment in ovarian cancer cell lines.

(2) the involvement of ERK1/2, a known ghrelin signalling pathway, in the ovarian cancer cells.
(3) the signalling pathways that mediate the effects of obestatin based on the results of proteomic analyses.
(4) the expression of obestatin in ovarian cancer tissues.

CHAPTER 2

Materials and methods

2.1 Peptides

Acylated ghrelin was obtained from Mimotopes (Clayton, Victoria, Australia) and amidated obestatin was obtained from Auspep (Parkville, Victoria, Australia), sequences in Table 2.1.

Table 2.1 Peptide sequences.

Peptide	Sequence
Acylated ghrelin	GSS(n-octanoyl)FLSPEHQRVQQRKESKKPPAKLQPR
Amidated obestatin	FNAPFDVGIKLSGVQYQQHSQAL-NH2

2.2 Cell lines and cell culture

In this study, experiments were performed using two serous ovarian cancer cell lines, SKOV3 and OV-MZ-6, (as serous cancers are the most common form of ovarian cancer), and functional effects compared to a normal representative cell line, hOSE 17.1. The SKOV3 ovarian cancer cell line and the hOSE 17.1 normal-derived human ovarian surface epithelial cell line (all obtained from the American Type Culture Collection, Rockville, Maryland, USA) and the OV-MZ-6 ovarian cancer cell line (a gift from Dr Daniela Lossener, Institute of Health & Biomedical Innovation, QUT) [510]. The SKOV3 ovarian cancer cell line is an epithelial ovarian serous adenocarcinoma cell line derived from ascitic fluid [511]. The hOSE 17.1 cell line is a normal human ovarian surface epithelial cell line immortalised with a retroviral vector expressing human papillomavirus oncogenes [512]. The human ovarian cancer cell line OV-MZ-6, is derived from malignant ascites from an advanced serous cystadeno ovarian cancer (International Federation of Gynecology and Obstetrics (FIGO) stage IV) [510].

The SKOV3 and hOSE 17.1 cell lines were routinely cultured in RPMI 1640 medium (Invitrogen, Mulgrave, Victoria, Australia), containing 10% (v/v) New Zealand Cosmic calf serum (FCS) (Thermo Fisher Scientific, Scoresby, Victoria, Australia), penicillin (100 units/mL) (Invitrogen) and streptomycin (100 µg/mL) (Invitrogen). The OV-MZ-6 cell line was maintained in Dulbecco´s Modified Eagle Medium (DMEM) liquid (high glucose, 4500 mg/L) (Invitrogen), containing 10%

(v/v) FCS (Thermo Fisher Scientific), 10 mM HEPES (Invitrogen), 0.550 mM L-arginine (Sigma-Aldrich), 0.272 mM L-asparagine (Sigma-Aldrich), 20 μg/ml gentamicin (Invitrogen), penicillin (100 units/mL) (Invitrogen) and streptomycin (100 μg/mL) (Invitrogen). All cell lines were maintained in an incubator at 37 ºC in 5% CO_2 and passaged at 70-80 % confluence by washing twice with phosphate buffered saline (PBS) and removal from the surface of the flask using 0.25% Trypsin/EDTA (Invitrogen). Cells were tested monthly for *Mycoplasma* contamination using RT-PCR (TaKaRa Bio Inc, Shiga, Japan) and found to be negative.

2.3 Quantitative real-time RT-PCR and RT-PCR of ovarian cancer tissues and cell lines

2.3.1 Statement of contribution for quantitative real-time RT-PCR and RT-PCR

The practical work described in this section was performed by Dr Inge Seim. Dr Inge Seim performed the quantitative real-time RT-PCR and RT-PCR of the tissues and cell lines while the candidate performed all other methods in this Chapter.

2.3.2 RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

To determine expression in cell lines, total RNA was harvested from 70% confluent monolayers of OV-MZ-6, SKOV3, and hOSE 17.1 cell lines using QIAshredder and RNEasy Plus Mini kits (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Total RNA (3 μg) from cell lines or stomach tissue (FirstChoice, Ambion, Austin, TX) was reverse transcribed using 10 units SuperScript III reverse transcriptase (Invitrogen) and 50 μM oligo$(dT)_{20}$ primers according to the manufacturer's instructions. The resulting single-stranded cDNA was treated with 2 U/μl ribonuclease H (Invitrogen) for 20 minutes at 37 °C in order to destroy complementary RNA and improve the sensitivity of the subsequent RT-PCRs [513, 514].

Expression of ghrelin, GHSR1a, GHSR1b and GOAT mRNA was assessed in the ovarian cell lines using RT-PCR. RT-PCRs for the housekeeping gene β_2 microglobulin were performed to check for genomic DNA contamination and to

Table 2.2 Primer sequences. Expected amplicon sizes (bp) and annealing temperatures (T_a) for RT-PCR.

Primer Pair	Primer Sequence (5'-3')	Size (bp)	T_a (°C)
Ghrelin			
Sense	GAGTCCAGCAGAGAAAGGAG	300	60
Antisense	ATGAGCGCTTCTAAACTTAGAG		
GHSR1a			
Sense	CGCTACTTCGCCATCTGCTT	542	60
Antisense	ATGGGGTTGATGGCAGCACT		
GHSR1b			
Sense	CGCTACTTCGCCATCTGCTT	433	60
Antisense	GGCACAGGGAGAGGATAGGA		
GHSR1a nested			
Sense	CTCTACAGTCTCATCGGCAG	126	60
Antisense	CTGTAGTGGTGTTTGCCTTC		
ß2 microglobulin			
Sense	TGAATTGCTATGTGTCTGGGT	248	55
Antisense	CCTCCATGATGCTGCTTACAT		

determine the integrity of the cDNA. Unless otherwise specified, all primers were designed using the Primer Express version 2.0 software (AB) and synthesised by Proligo (Armidale, NSW, Australia) (Table 2.2). Commercially available primers were used for GOAT assays (*MBOAT4*, SABiosciences). RT-PCRs were performed in a total reaction volume of 50 µl using 10 U Platinum *Taq* Polymerase (Invitrogen) according to the manufacturer's instructions. A nested RT-PCR was performed for the detection of GHSR1a. Briefly, PCR products from the first GHSR1a RT-PCR were diluted 1/100 in water and used in a second PCR with nested GHSR1a primers (Table 2.2). In RT-PCR assays for GHSR1b [186], which contains intronic sequence, RNA was treated with *DNase I* and a no-reverse transcriptase control (RT-) was employed to ensure that only mRNA (and not genomic DNA) was amplified. Negative controls, with water substituted for template, were also performed for all PCRs. RT-PCRs were performed, as outlined in Table 2.2, with a PTC-200 thermal cycler (MJ Research, Watertown, MA). Sequencing was performed in order to confirm the identity of the RT-PCR products. The reaction products were purified using the PCR MinElute kit (QIAGEN), subcloned into *pCR-XL-TOPO* (Invitrogen), transformed into One Shot MAX Efficiency *DH5α-T1R* chemically-competent cells (Invitrogen), purified using a miniprep kit (QIAGEN) and sequenced at the Australian Genome Research Facility (AGRF, Brisbane, Australia) using BigDye III (Applied Biosystems, Victoria, Australia).

2.3.3 Quantitative real-time RT-PCR of clinical specimens

Real-time RT-PCR was used to detect ghrelin (*GHRL)* and *GHSR1a* mRNA levels in a range of samples of normal ovary and from ovarian carcinoma samples using an OriGene TissueScan qPCR Ovarian Cancer panel (panel II, OriGene, Rockville, MD). This array consists of pathologist-verified samples from eight non-tumour ovarian tissues and 40 different ovarian tumours. The panel consisted of RNA from patients with stage I, II and III ovarian cancer. Ages of tissue donors ranged from 33 to 91 years.

Real-time RT-PCRs were performed using the AB 7000 sequence detection system (AB), in a total reaction volume of 20 µl. Platinum Quantitative PCR SuperMix-UDG w/ROX (Invitrogen) was used to detect *GHRL* and *GHSR1a* expression, while the RT-PCRs for the β-actin housekeeping gene (*ACTNB*) and *MBOAT4*/GOAT were

performed using 2 × SYBR green master mix (AB). β-actin primers were supplied with the cDNA panel (OriGene), and primers for *MBOAT4*/GOAT were purchased from SABiosciences (Frederick, MD). Primers and TaqMan probes for *GHRL* (Hs01074051_g1, spanning the ghrelin-peptide coding exons and Hs01074053_m1, spanning the obestatin-peptide coding exons), and *GHSR1a* (Hs01026313_m1) were obtained from Applied Biosystems (AB). Each gene was evaluated using two separate array plates and normalised against β-actin. Fold changes were quantified as $2^{-(\Delta Ct\ sample-\Delta Ct\ control)}$, as described previously [515]. Negative control RT-PCRs were also performed where template was substituted with water.

2.4 Cell proliferation assay

Proliferation was measured using 2 different types of assays to confirm that results obtained were due to a difference in cell number and not just changes in metabolism. Cell proliferation was measured using a WST-1 (Roche, Nonnenwald, Penzberg, Germany) assay, which is based on the cleavage of the WST-1 tetrazolium salt by mitochondrial dehydrogenases in viable cells, and CyQuant (Invitrogen), which quantifies cell DNA content as a direct representation of cell number.

In order to determine the optimal cell density for these assays, a seeding density test was performed by seeding a range of cell numbers into a 96-well plate and their percent confluence was observed under the phase contrast microscope over 96 h. Standard curves of cell number were also performed to confirm that cell number was proportional to absorbance or luminescence. Cells (1×10^4 cells/mL for SKOV3 and OV-MZ-6 and 5×10^4 cells/mL for the hOSE 17.1) were plated in 200 µL phenol red free medium with 10% FCS (v/v) in each well of duplicate 96-well plates (black and clear) and incubated at 37ºC for a total of 96 h. After 24 h, cells were treated with a range of ghrelin or obestatin concentrations (0.1, 1.0, 10, 100, 1000 nM) or no treatment controls (0 nM) for 3 days (with ghrelin being replenished every 24 hours). Cells were then treated with WST-1 reagent (Roche) for 1.5 h in phenol red free medium, according to the manufacturer's instructions, and absorbance was determined (450/650 nm) using a Benchmark Plus Microplate Spectrophotometer System (BioRad, Regents Park, NSW, Australia). CyQuant NF assays for cell DNA content (Invitrogen) were performed in black plastic plates (PerkinElmer Life Sciences, Melbourne, Vic, Australia) according to the manufacturer's instructions and fluorescence (520 nm) was measured after excitation at 480 nm using a

microplate reader PolarSTAR Optima (BMG Labtech, Victoria, Australia). Each treatment (and vehicle only controls) was performed using 16 replicate wells and each experiment was performed independently at least 3 times.

2.5 Transwell migration and invasion assay

Cell migration and invasion assay were performed using a modified Transwell protocol (Fig 2.1) [516]. Cells were split into T25 flasks (Nunc Nalge, Thermo Fisher Scientific, Victoria, Australia), and 24 h after seeding they were treated with 10 or 100 nM ghrelin, or obestatin, or medium only for 48 h. Migration and invasion assays were performed in 24-well plates (Nunc Nagle) with Transwell^{TM} cell culture inserts with 8 µm pores (Falcon; BD Biosciences, NSW, Australia) and 10% FCS was added to the lower well as a chemoattractant. For invasion assays, the upper surfaces of the inserts were coated with growth factor-reduced synthetic extracellular matrix (ECM) Matrigel (BD Biosciences), where 50 uL Matrigel (10ug/ml) was overlaid with 200 uL ice-cold PBS and left in the incubator at 37°C overnight to set. Two hundred thousand cells from each cell line were seeded in 400 µL serum-free medium containing 0.1% (w/v) bovine serum albumin (BSA) with or without ghrelin or obestatin (0, 10 nM and 100 nM) into the top chamber of the well and incubated for 24 h (for migration assays), or 48-72 h (for invasion assays). Control inserts containing 400 µL serum-free medium with 0.1% (w/v) BSA were also set up to demonstrate any background staining. After these incubation periods, cells remaining on the upper surface of the insert were removed using a cotton bud. To quantify the number of cells that migrated, or invaded cells on the underside of the insert, cells were fixed with 100% ice-cold methanol and stained with 1% (w/v) crystal violet. After washing, the stain was extracted from the cells using 10% (v/v) acetic acid and absorbance (595 nm) measured. For each experiment 3 replicates per treatment were performed and the experiments were repeated independently 3 times. Standard curves for cell number were also performed in 96 well plates with the same fixing and staining protocol to confirm that cell number was proportional to absorbance.

2.6 Attachment assay

Attachment assays were performed using a modified version of protocols reported by Festuccia *et al*. (1999) and Romanov *et al*. (1999) [517, 518]. Cells were pre-treated with 0, 10 and 100 nM ghrelin, or obestatin for 2 days prior to the attachment assay.

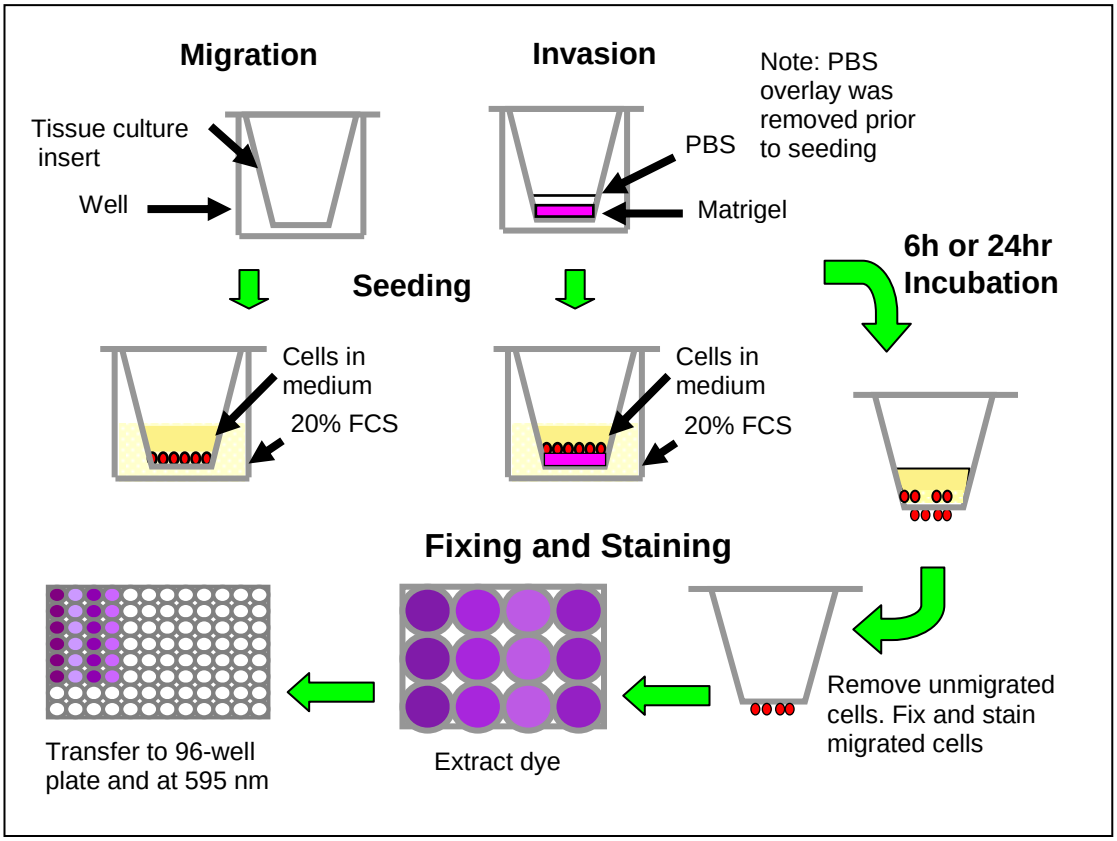

Figure 2.1 Schematic overview of the migration and invasion assays.

Plates with 96 wells were coated overnight with purified ECM molecules and basement membrane components, fibronectin (BD Biosciences), vitronectin (BD Biosciences), collagen I (Calbiochem; EMD Biosciences, Merck PTY, Kilsyth, Victoria, Australia) or collagen IV (Calbiochem; EMD Biosciences, Merck PTY), diluted to a concentration of 10 µg/mL (50 µL/well) in RPMI, or DMEM/F12 (medium without additives). Matrix solution was overlaid with ice-cold PBS to prevent the coating from drying out and to ensure an even coating. After overnight incubation, PBS was removed and non-specific binding sites were blocked with 1% (w/v) BSA. Cells from each cell line (20 000 cells per well) were seeded in 100 µL serum-free medium with 0.1% (w/v) BSA into each well of the matrix coated plate or in a BSA blocked only plate (for background adherence) and incubated for 1 h at 37 ^{o}C. A third plate with no coating or blocking was also seeded with cells (including 5% FCS) and incubated for 5 h to ensure total cell attachment to estimate the absorbance of total cell adherence (100% plate). After 1h, or 5 h, media was aspirated from the wells and the cells carefully washed twice with PBS to remove any non-adhered cells. Adherence was then determined by staining the cells using CyQuant, as per the manufacturer's instructions. Results were corrected for background attachment and expressed as a percentage of adhesion to the 100% plate, a representation of 100% potential adhesion. The assay was performed in triplicate with 3 independent replicates of each ECM molecule per assay.

2.7 Western immunoblotting using anti-phosphorylation antibodies

Western analysis was performed using an antibody for activated (phosphorylated) ERK1/2 to determine if ghrelin treatment activates this signalling pathway. Ovarian cells were seeded at 3 x10^5 cells/well in 6-well plates. After 48h, cells were incubated in phenol red free medium without FCS for 24 h. To determine an appropriate time-point to investigate ERK1/2 signalling, cells were treated with ghrelin or obestatin (0, 10 or 100 nM) and incubated for 0, 5, 15, 30, 45, or 60 min and whole cell lysates were collected for Western analysis. A time-point at which strong stimulation of ERK1/2 occurred was determined to be 15min for the SKOV3 ovarian cancer cell line and 30min for the OV-MZ-6 cell line. This is consistent with another study in porcine ovarian co-culture cells that observed maximum stimulation of phospho-ERK 1/2 levels after 15 min of cell incubation [509]. To determine if the ERK1/2 signalling is dose dependent, cells were also treated with a range of ghrelin

or obestatin concentrations (0, 0.1, 1, 10,100 and 1000nM) at the timepoint where maximal activation occurred for each cell line.

Whole cell lysates were collected using lysis buffer consisting of 20 mM Tris, pH 7.5, 150 mM NaCl, 1% (w/v) Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM beta-glycerophosphate, 50 nM NaF, complete EDTA-free protease inhibitor cocktail (Roche) and Phosphate Inhibitor Cocktail 2 (Sigma-Aldrich). Lysates were vortexed, and centrifuged (10000 *x* g for 20 min at 4 ºC) to remove cell debris and protein content was estimated using a biciconinic acid kit (BCA), according to the manufacturer's instructions (Pierce Protein Research Products, Thermo Fisher Scientific, Victoria, Australia). Protein lysate samples (30 μg) were boiled in loading buffer (0.1 M Tris, pH 6.8, 4% (v/v) SDS, 20% (v/v) glycerol, 0.2% (w/v) bromophenol blue, 10 mM DTT) for 5 min, and samples were electrophoresed using 11% (w/v) SDS polyacrylamide reducing gels in Laemmli buffer (0.0255 M Tris, 0.25 M glycine, 0.1% (w/v) SDS, pH 8.3). The proteins were transferred onto nitrocellulose membranes (Schleicher and Schuell; Medos, Mt Waverley, Victoria, Australia) in Tris-Glycine buffer (25 mM Tris, 40mM Glycine, 10% (v/v) methanol) at 350 mA for 90 mins at 4 ºC. The membrane was blocked with 5% (w/v) skim milk or 2.5% (w/v) BSA (Sigma) in TBS-Tween 20 (0.05 M Tris, 0.25 M NaCl, pH 7.4, 0.05% (v/v) Tween-20) for 1 h at room temperature to prevent non-specific binding.

Western immunoblotting for signalling molecules was performed using primary anti-human antibodies (Cell Signalling, Genesearch, Arundel, Qld, Australia), mouse anti-phospho-p44/42 MAPK (Thr202/ Tyr204) (E10) and rabbit anti-p44/42 MAP Kinase antibodies. Anti-phosphorylated ERK1/2 antibodies were used at a concentration of 1:2000 and antibodies against total ERK1/2 protein at 1:5000. Mouse anti-Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH, MAB374, Chemicon, 1:20000) was used to demonstrate even protein loading. The nitrocellulose membranes were incubated overnight with primary antibody at 4 ºC, (diluted in either 5% (w/v) skim milk or 2.5% (w/v) BSA (Sigma) in TBS-Tween 20). Membranes were washed with TBS-Tween 20, then incubated for an hour at 1:5000 with either goat anti-rabbit (Thermo Fisher Scientific, Victoria, Australia), or goat anti-mouse (Pierce) horseradish peroxidase-conjugated IgG as the secondary antibody. After further washing in TBS-Tween 20 for 5 x 5 min, blots were

incubated with West Femto chemiluminescent substrate (Pierce) for 5 min and exposed to X-ray film which was developed using a Curix 60 automatic developer (Agfa, Stafford, QLD, Australia).

2.7.1 Densitometry

Western immunoblots were quantitated by scanning the X-ray film and analysing images using the Image J software program [519]. The Image J software generates a profile plot based on the intensity of the band of interest, quantitates the area under the curve and exports the values to Excel. These values were used for comparisons of band intensity using the GraphPad Prism v.5.01 software (GraphPad Software, Inc., San Diego, CA).

2.8 Statistics

Statistical analyses were undertaken using GraphPad Prism v.5.01 software (GraphPad Software, Inc., San Diego, CA). For proliferation, migration, invasion and attachment assays, statistical significance was determined using one-way ANOVA, followed by Tukey's *post hoc* test (with $P < 0.05$ considered to be statistically significant).

CHAPTER 3

Ghrelin stimulates cell migration in ovarian cancer cell lines

3.1 Introduction

Ghrelin is a multifunctional hormone with a wide range of effects, including the potent stimulation of appetite and a role in regulating gut motility, metabolism and energy balance [1, 125, 126]. Ghrelin also has roles in regulating the reproductive, cardiovascular and immune systems and has effects on sleep, memory and mood [4, 337]. The ghrelin axis has potential as a therapeutic target for inflammation and heart disease [4] and may be useful in treating cachexia, which is associated with a number of diseases including cancer [360, 520]. Ghrelin also appears to have a role in cancer and stimulates processes related to cancer progression including cell proliferation [17, 25, 38-40, 47-49, 54, 56, 57], and cell migration and invasion [48, 54] and it also has anti-apoptotic effects [47, 49, 52].

Ghrelin circulates in the plasma as acylated ghrelin, and as a non-octanoylated form, desacyl ghrelin [1]. The octanoylation of ghrelin is mediated by the enzyme, ghrelin *O*-acyltransferase (GOAT) [2, 3, 122]. Octanoylated ghrelin acts through the type 1a isoform of the growth hormone secretagogue receptor, GHSR1a, while GHSR1b is a truncated isoform, does not bind ghrelin and was originally thought to be non-functional [1, 185]. There is evidence, however, that the GHSR1b isoform could regulate the expression and action of the GHSR1a [521-523]. Ghrelin and desacyl ghrelin are also thought to act through an alternative ghrelin receptor, as a number of studies have demonstrated that they function in the absence of GHSR1a expression [38, 44, 50, 51]. Although originally thought to be an inactive form of ghrelin, desacyl ghrelin has a number of functions in common with ghrelin, such as the modulation of proliferation [38-40, 56, 57]. Desacyl ghrelin can also have the opposite effect to ghrelin, for example it inhibits gastric emptying, while ghrelin promotes emptying [204, 261-263].

Ghrelin has also been demonstrated to have functional effects in cancer, and the most studied of these is its role in proliferation. It stimulates cell proliferation in a number of cancer cell lines including prostate cancer [39], breast cancer [17], and endometrial cancer cell lines [49], although ghrelin inhibits cell proliferation in adrenocortical carcinoma [53], thyroid carcinoma [44, 56] and prostate cancer [38] cell lines. Ghrelin treatment has been shown to stimulate cell proliferation, acting through the ERK1/2 MAPK (mitogen-activated protein kinase) pathway in a number

of cell lines, including prostate cancer [39, 40], pituitary tumour cells [57] and hepatoma cell lines [25]. Inhibition of the ERK1/2 signalling pathway, using ERK1/2 inhibitors, reversed ghrelin-stimulated proliferation in prostate cancer cell lines [40].

It has also been recently demonstrated that ghrelin may stimulate cell migration in some cancer cell types [16, 48, 54, 55]. Studies that have used exogenous ghrelin treatments, inhibited endogenous ghrelin with a ghrelin antibody, used antagonists for GHSR1a, or inhibited ghrelin signalling pathways have demonstrated that ghrelin stimulates cell migration, or induces a motile phenotype in a number of cell types [16, 48, 54, 55, 190]. Ghrelin stimulates migration in some normal cell types, including human microvascular endothelial cells (HMVEC) [174], and cardiac microvascular endothelial cells (CMECs) [171]. Although a number of studies have shown that ghrelin stimulates migration, there have been few studies investigating the signalling pathways that mediate this response and there is little consensus. In a study of glioma cells, ghrelin-mediated migration was attenuated by inhibition of the calcium/calmodulin-dependent kinase II (CaMKII), AMP-activated protein kinase (AMPK), and nuclear factor-kappaB (NF-κB) signalling pathways [55]. In pancreatic adenocarcinoma cell lines, increased motility, in addition to increased invasiveness and proliferation, coincided with increased phosphorylation of Akt [54]. In this study, however, inhibition of Akt phosphorylation downregulated proliferation and invasion, but not cell motility. Cell motility and invasiveness were suppressed by Wortmannin, a phosphoinositide 3 kinase (PI3K) inhibitor [54]. Motility of astrocytoma cell lines was mediated by an increase in intracellular calcium mobilisation and PKC activation [16]. In normal cell types, ghrelin-stimulated migration, proliferation and angiogenesis in CMECs and in HMVECs was accompanied by increased ERK1/2 signalling [172, 174].

Currently, the role of ghrelin in ovarian cancer is unknown. Ghrelin treatment has previously been shown to stimulate ERK1/2 activation in the SKOV3 ovarian cancer cell line and this was linked to an anti-apoptotic effect when apoptosis was induced by Paris Saponin I [524]. Interestingly, serum acylated ghrelin levels were elevated in a small study of patients with ovarian cancer and benign ovarian tumours [114], indicating that ghrelin could be a useful serum marker for ovarian cancer, or play a role in ovarian cancer development. There were a number of limitations to this study,

however, including small sample size. In a recent immunohistochemical study, ghrelin expression was demonstrated in a range of malignant serous ovarian tumours and was elevated compared to benign ovarian tumour specimens [380]. It is unclear if elevated plasma levels could be correlated with increased levels of expression in ovarian cancer, however, it is possible that ghrelin expressed by ovarian cancer cells could act as an autocrine/paracrine factor. GHSR1a expression has been demonstrated immunohistochemically in a number of ovarian pathologies, including inclusion cysts from the ovarian surface epithelium and in benign serous ovarian tumours [45]. GHSR1b expression has not been investigated in ovarian cancer, however, it has been demonstrated in other cancers including breast [17], lung [29] and colon [19, 20, 48] tumours.

Epithelial ovarian cancers, derived from the ovarian surface epithelium, are the most fatal of all gynaecological cancers and they are frequently highly metastatic [525, 526]. Ovarian cancer presents with few symptoms, particularly during initial tumour development, and, therefore, ovarian cancer is difficult to diagnose at a low tumour grade [66]. This contributes to the poor prognosis for many ovarian cancer patients, and two thirds of ovarian cancer patients are not diagnosed until the late stages of cancer development, (at either stage III or IV) [61]. The development of new markers for the early diagnosis of ovarian cancer and better adjunct treatments would lead to better patient outcomes.

In this study we examined the expression of ghrelin, GHSR1a, GHSR1b and GOAT in ovarian cancer cell lines and tissues. We investigated the effect of exogenous ghrelin treatment on cell proliferation, migration, invasion and attachment, which are key processes in cancer progression in ovarian cancer cell lines [393]. We also investigated whether ghrelin stimulated the ERK1/2 signalling pathway in the ovarian cancer cell lines.

3.2 Materials and methods

3.2.1 Statement of contribution

The practical work described in this Chapter was performed through a collaboration between the candidate, Carina Walpole, and Dr Inge Seim. Dr Inge Seim performed

the quantitative real time RT-PCR and RT-PCR of the tissues and cell lines (Results 3.3.1) while the candidate performed all other experiments.

3.2.2 Cell lines and Cell culture

In this study assays were performed using the SKOV3 human epithelial ovarian serous adenocarcinoma cell line [511], the hOSE 17.1 human normal ovarian surface epithelium-derived cell line [512] and the OV-MZ-6 human epithelial ovarian serous adenocarcinoma cell line [510]. The SKOV3, OV-MZ-6 and hOSE 17.1 cell lines used in this study were propagated as described in the General Methods, Chapter 2.1.

3.2.3 Expression of ghrelin axis components at an mRNA level in ovarian cell line and tissues.

3.2.3.1 Quantitative real-time RT-PCR of tissue mRNA

Real-time RT-PCR was performed to quantify expression levels of ghrelin (*GHRL)*, *GHSR1a* and *GOAT (MBOAT4*) mRNA expression (as described in Chapter 2.3.3) using cDNA from normal ovary and from ovarian cancer patients using tissue cancer panels (OriGene TissueScan qPCR Ovarian Cancer panel II, OriGene, Rockville, MD). This array consists of pathologist-verified samples from eight non-tumour ovarian tissues and 40 different ovarian tumours.

3.2.3.2 RT-PCR of cell line mRNA

RT-PCR was used to determine the expression of components of the ghrelin axis (Table 3.1) in the normal and cancer ovarian cell lines. RNA was extracted and cDNA synthesised from SKOV3 and OV-MZ-6 cancer cell lines and normal hOSE 17.1 cell lines (as described in Chapter 2.3). RT-PCR was performed using cDNA from the normal ovary and from ovarian cancer cells (as described in Chapter 2.3). Stomach tissue was used as a positive control and negative control RT-PCRs were also performed where template was substituted with water.

3.2.4 Cell proliferation assays

WST-1 and CyQuant cell proliferation assays were performed as described (Chapter 2.4) using the SKOV3 and OV-MZ-6 ovarian cancer cell lines and hOSE 17.1 normal-derived cell line with the addition of a range of acylated ghrelin

concentrations (0, 0.1, 1, 10, 100 and 1000 nM) and no treatment controls (0 nM). Each treatment (and control) was performed using 16 replicate wells and each experiment was performed independently at least 3 times.

3.2.5 Cell migration and invasion assays

Migration and invasion assays were performed (as described in Chapter 2.5) using the SKOV3 and OV-MZ-6 ovarian cancer cell lines and the hOSE 17.1 normal-derived ovarian cell line. For invasion assays, the upper surfaces of the inserts were coated with growth factor-reduced extracellular matrix (ECM) Matrigel (BD Biosciences). Cells were seeded in the upper chamber of the Transwell in FCS-free 0.1% BSA growth media, with or without ghrelin (0, 10 and 100 nM). Additional controls were performed to determine background staining using inserts without cells, containing medium only. For each experiment, 3 replicates per treatment were performed and the experiments were repeated independently 3 times.

3.2.6 Attachment assays

To assess whether ghrelin treatment had any effect on cellular attachment, the hOSE 17.1 normal ovarian cell line and OV-MZ-6 and SKOV3 ovarian cancer cell lines were treated and then tested for their ability to attach to extracellular matrix (ECM) proteins fibronectin, vitronectin, collagen I and collagen IV. Attachment assays were performed as described in Chapter 2.6. The assay was performed in triplicate with 3 independent replicates of each ECM molecule per assay.

3.2.7 Western immunoblotting using anti-active antibodies for ERK1/2

Western analysis was performed as previously described (Chapter 2.7) using an anti-active antibody for phosphorylated ERK1/2 to determine if ghrelin treatment activates this signalling pathway. To determine if ERK1/2 signalling is dose dependent, cells were treated with ghrelin (0, 0.1, 1, 10,100 and 1000 nM).

3.2.7 Statistics

Statistical analyses (one way ANOVA with *post hoc* tests) were undertaken using Graphpad as described in Chapter 2.8.

3.3 Results

3.3.1 Expression of the ghrelin axis in normal ovary, ovarian tumour tissue and in ovarian cell lines

Using quantitative, real-time RT-PCR analysis we have demonstrated that ghrelin (Fig. 3.1) and the enzyme that octanoylates ghrelin, GOAT, are expressed in normal ovarian tissue and in a range of ovarian cancer tissues (Fig. 3.2). We investigated expression in a range of cancer stages (I-IV), where increasing stage reflects the transition of cells from a confined tumour to a metastasised cancer [65]. There was no clear correlation between cancer stage (stages I-IV) and the level of ghrelin or GOAT mRNA expression, although more than two times the level of expression of ghrelin and/or GOAT was observed in 7/34 ovarian cancer samples, compared to normal tissue. Using primers in exons 1 and 2 of the GHSR, expression of ghrelin receptor (GHSR1a) mRNA was not observed in any of the normal or cancer ovarian tissues examined using the Origene panel (data not shown).

In order to investigate the expression of ghrelin in ovarian cell lines and to characterise a model for further functional analyses, we examined the mRNA expression of ghrelin, GOAT and the ghrelin receptor isoforms, GHSR1a and GHSR1b, using RT-PCR. Full-length preproghrelin (exon 1 to 4) and GOAT were expressed at the mRNA level in the SKOV3 and OV-MZ-6 ovarian cancer cell lines and in the hOSE 17.1 normal ovary derived cell line, giving RT-PCR products of the expected sizes (Fig. 3.3). In addition, the exon 3 deleted preproghrelin isoform [17] was also detected by RT-PCR in the SKOV3 ovarian cancer cell line (191 bp product, Fig. 3.3). A 433 bp product for GHSR 1b mRNA was expressed in the OV-MZ-6 and SKOV3 ovarian cancer cell lines, but not in the hOSE 17.1 normal ovary-derived cell line (Fig. 3.3). As expected, the no-RT control failed to amplify RT-PCR product, demonstrating that the GHSR1b cDNA was not amplified from contaminating DNA. GHSR1a mRNA was not expressed in any of the cell lines examined, but a product of the expected size was observed in the positive control from stomach tissue (Fig. 3.3). Positive controls gave bands of the expected size for β_2 microglobulin, demonstrating the integrity of the cDNA used in these assays for the three cell lines and the no-template, negative controls failed to amplify product. The identity of RT-PCR products was confirmed by cDNA sequencing.

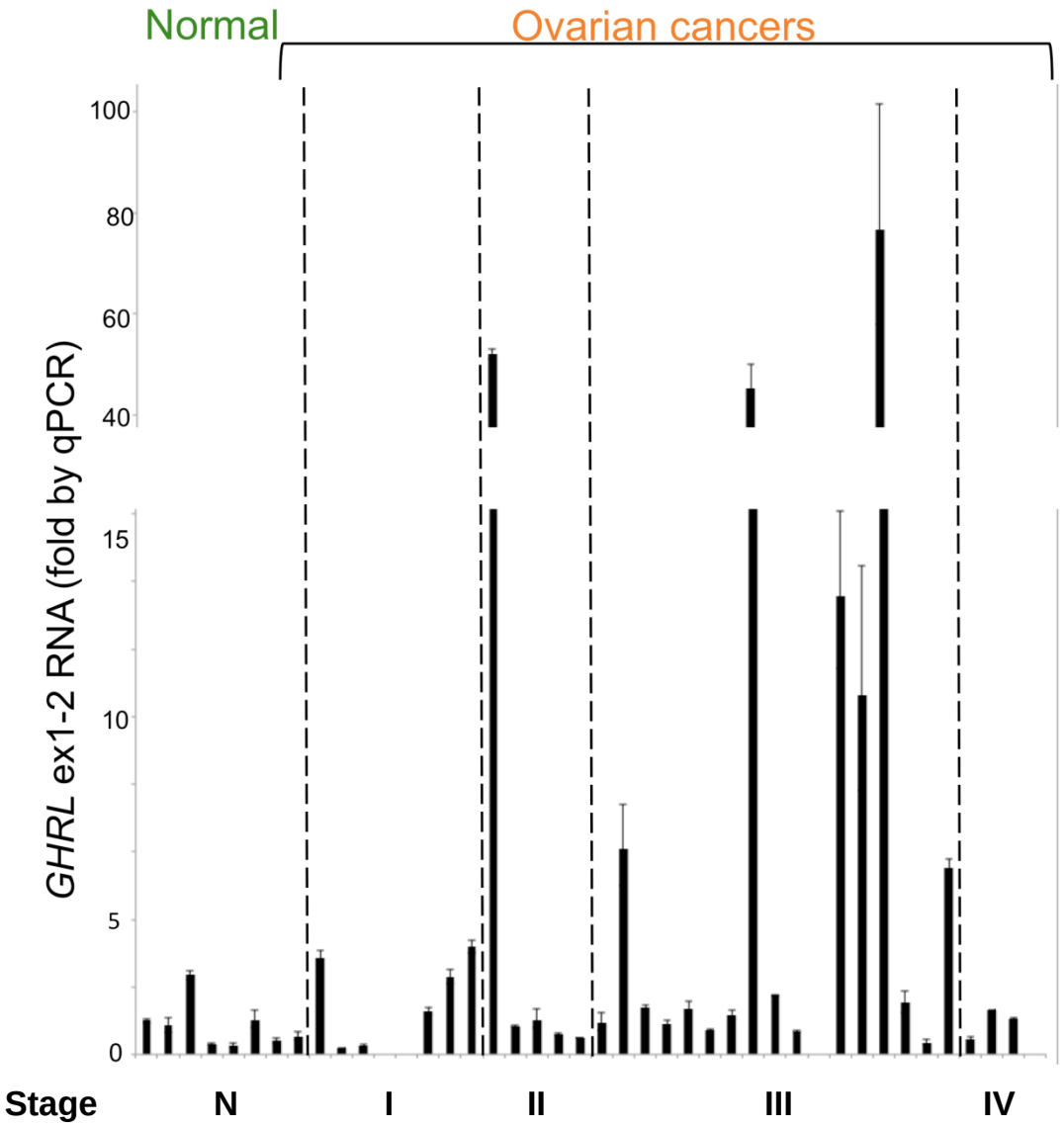

Figure 3.1 Quantitative RT-PCR analysis of *GHRL* exon 1-2 expression in normal and stage I to IV ovarian cancer. Relative expression of ghrelin (*GHRL* exon 1-2) mRNA in normal ovary (N) and in stages I-IV of ovarian cancer was determined using quantitative real-time RT-PCR using an Origene TissueScan ovarian cancer panel. Data were normalised to β-actin, quantified using $2^{-(\Delta Ct\ sample-\Delta Ct\ control)}$, and represented as fold change relative to expression of transcripts in a normal ovarian tissue sample (1.0). Bars indicate mean +/- SEM.

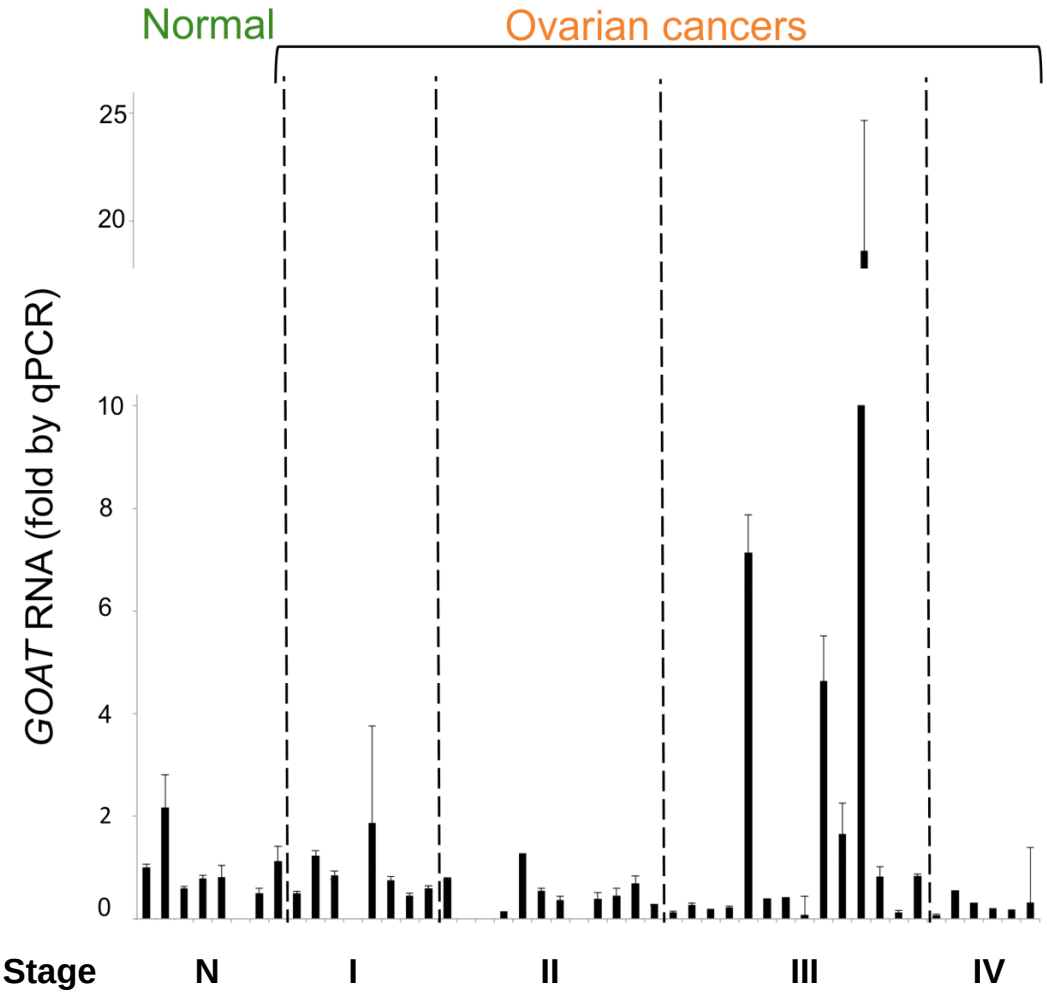

Figure 3.2 Quantitative RT-PCR analysis of GOAT expression in normal and stage I to IV ovarian cancer. Relative expression of GOAT (*MBOAT4*) mRNA transcripts in normal ovary (N) and ovarian cancer samples (stages I-IV) was determined using quantitative real-time RT-PCR using an Origene TissueScan panel. Data were normalised to β-actin and are represented as fold changes relative to expression of transcripts in a normal ovarian tissue sample (1.0) and quantified using the equation $2^{-(\Delta Ct\ sample-\Delta Ct\ control)}$. Negative control RT-PCRs were also performed where template was substituted with water (data not shown). Bars indicate mean +/- SEM.

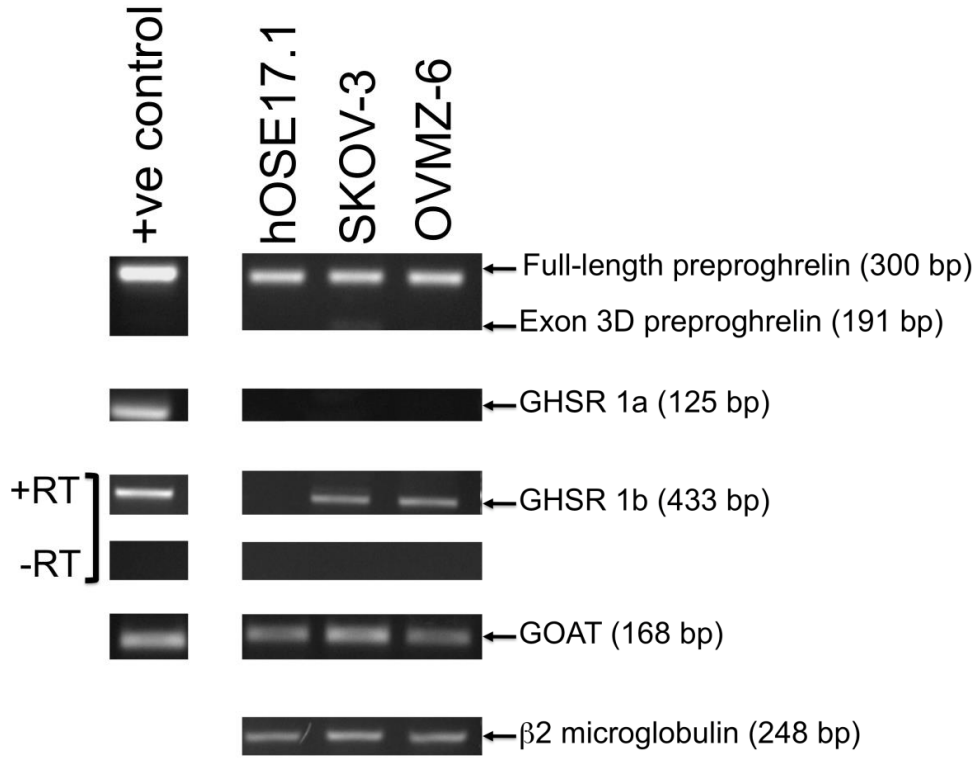

Figure 3.3 Preproghrelin and GOAT (*MBOAT4*), but not GHSR1a, are expressed at the mRNA level in the OV-MZ-6 and SKOV3 ovarian cancer cell lines and in the hOSE 17.1 normal derived ovarian cell line. Ethidium bromide stained agarose gels demonstrating RT-PCR products of the expected size for ghrelin (300 bp), GHSR1a (125 bp), GHSR1b (433 bp) and GOAT (168 bp). The GHSR1b mRNA isoform is expressed in the SKOV3 and OV-MZ-6 cancer cell lines, but not in the normal-derived hOSE 17.1 cell line. An exon 3 deleted preproghrelin isoform, represented by a 191 bp product, is also expressed in the SKOV3 ovarian cancer cell line (and may be seen as a very faint band). The identity of these products was confirmed by cDNA sequencing. Human stomach tissue RNA was used as a positive control (+ve) for ghrelin, GHSR1a and GOAT expression, while the PC3 prostate cancer cell line was the positive control tissue for GHSR1b. As the GHSR1b isoform contains intronic sequence, RT-PCR was performed using RNA samples that were not reverse transcribed (+/-RT) as a control for genomic DNA contamination. Negative controls, where template was replaced with water, were used for all RT-PCRs and no products were amplified. RT-PCRs for the β_2 microglobulin housekeeping gene were performed to ensure that cDNA was present in the reactions. M = molecular weight marker.

3.3.2 Cell migration assays

As ghrelin has also been demonstrated to stimulate cell migration in a number of cell types [16, 48, 54, 55, 171, 174], we investigated the role of ghrelin in stimulating cell migration of the SKOV3 and OV-MZ-6 ovarian cancer cell lines and the hOSE 17.1 normal ovary-derived cell line. Ghrelin treatment (100 nM) significantly increased cell migration across the membrane in Transwell assays in the SKOV3 and OV-MZ-6 ovarian cancer cell lines after 24 hours ($P < 0.05$) compared to untreated controls. Ghrelin treatment led to an increase of 3.92 +/- 2.15 fold in the SKOV3 cell line and 1.77 +/- 0.30 fold in the OV-MZ-6 cell line compared to the control (Fig. 3.4). No significant difference was seen with the 10 nM ghrelin treatment in these cell lines, and ghrelin had no significant effect on cell migration in the hOSE 17.1 normal ovarian surface epithelial-derived cell line.

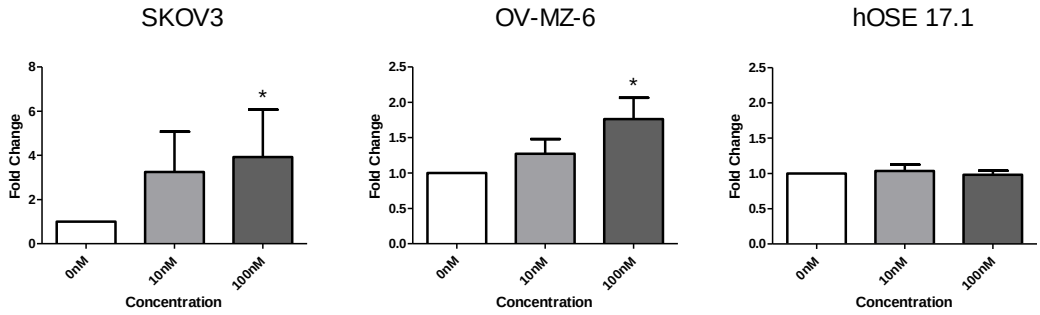

Figure 3.4 Ghrelin stimulates cell migration in ovarian cancer cell lines.
Transwell migration assays were performed with SKOV3 and OV-MZ-6 ovarian cancer cell lines and compared to the normal-derived ovarian surface epithelial cell line hOSE 17.1. Ghrelin (100 nM) stimulated cell migration in the SKOV3 and OV-MZ-6 ovarian cancer cell lines, but not in the hOSE 17.1 normal ovary-derived cell line in Transwell assays with 8 µm pore membranes over 24 h. Migration was assessed by staining migrated cells with 1% crystal violet, and absorbance measured as a representation of the number of migrated cells. Graph represents mean fold change +/- SEM. *$P < 0.05$, assessed by Tukey's *post hoc* test, compared to 0 nM control. n = 3 repeat experiments with 3 replicates per experiment.

3.3.3 Cell invasion assays

As we demonstrated that treatment with ghrelin increases migration in ovarian cancer cell lines, we performed Transwell invasion assays to determine if ghrelin also alters the rate of cell invasion through Matrigel. No significant difference in invasion was observed in the three cell lines with ghrelin treatment (Fig. 3.5).

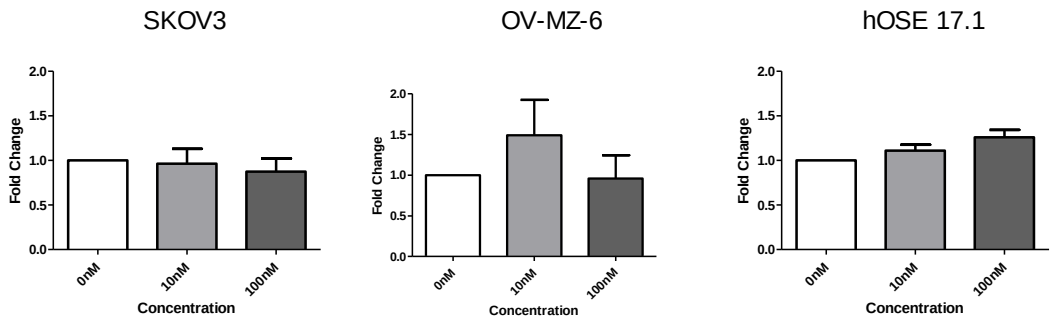

Figure 3.5 Ghrelin treatment did not alter cell invasion in the SKOV3 and OV-MZ-6 ovarian cancer cell lines and the hOSE 17.1 normal ovarian epithelial-derived cell line in Transwell invasion assays (where the membrane was overlaid with Matrigel). Transwell assays were performed over 48 h (SKOV3 and OV-MZ-6) or 72 h (hOSE 17.1). Mean absorbance +/- SEM. *P <0.05 compared to 0 nM control using one way ANOVA and Tukey's *post hoc* comparisons. n = 3 repeat experiments with 3 replicates per experiment.

3.3.4 Cell proliferation assays

As we have previously demonstrated that ghrelin stimulates cell proliferation in a range of cell lines, including breast [17], prostate [39] and endometrial cancer [49], we investigated the effect of a range of concentrations of ghrelin on cell proliferation in ovarian cell lines, using both a metabolic-based assay, WST-1, and a DNA-based assay, CyQuant. In contrast to our studies in other cell lines, no statistically significant changes in proliferation were observed in the SKOV3 or in the OV-MZ-6 ovarian cancer cell line or in the hOSE 17.1 cell line (Fig. 3.6) using either assay method.

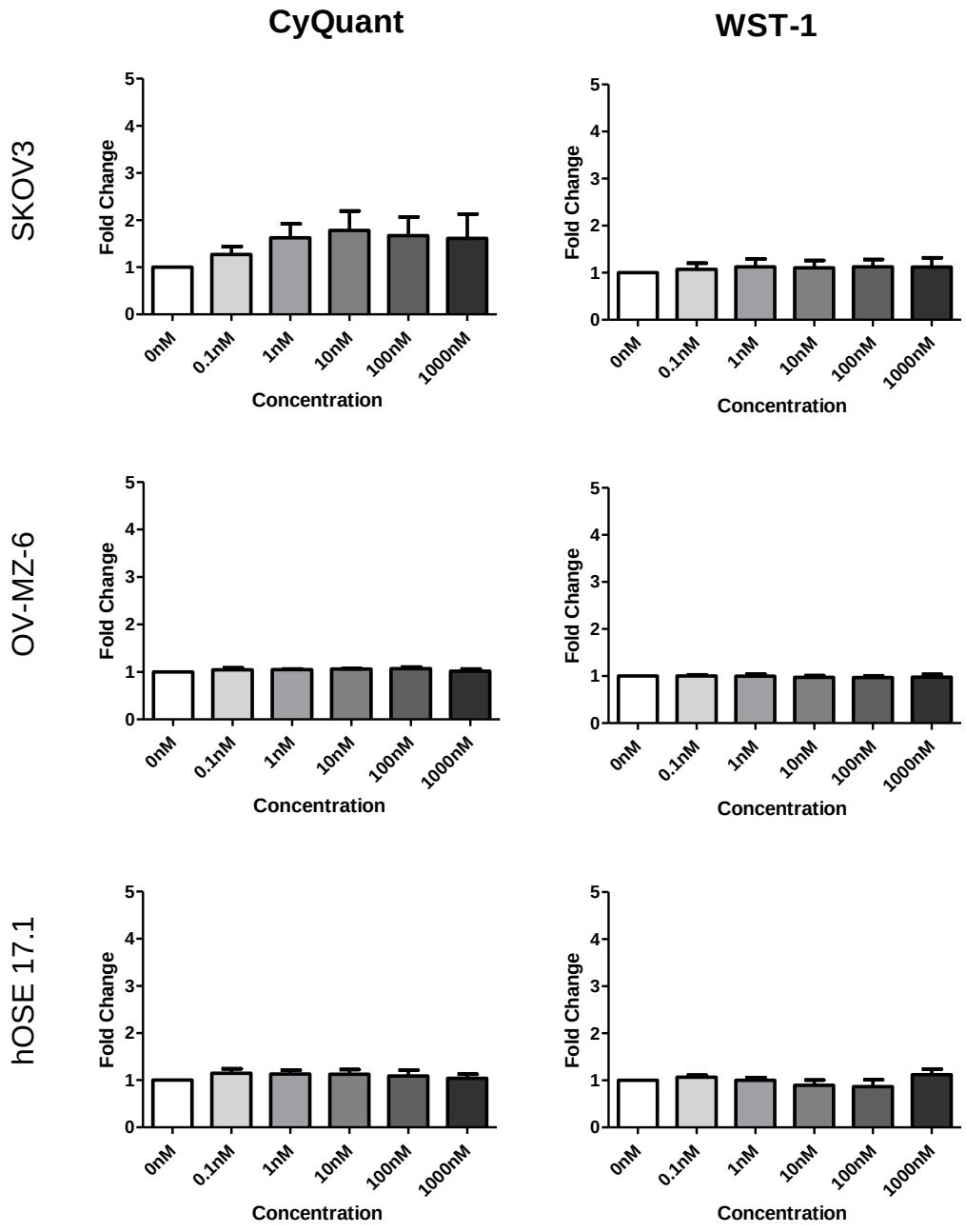

Figure 3.6 Ghrelin treatment does not stimulate cell proliferation in normal, or ovarian cancer cell lines. Histograms demonstrate that ghrelin (0-1000 nM) does not stimulate cell proliferation assays in the SKOV3 and OV-MZ-6 ovarian cancer cell line and the hOSE 17.1 ovarian cell line. Cell proliferation was measured using a CyQuant (DNA- based) assay and WST-1 (metabolic-based) cell proliferation assays and expressed as fold change over the untreated control (0 nM). No statistically significant differences were observed (one way ANOVA). Histograms represent combined data from n = 3 repeats with 16 replicates per independent experiment. Bars represent mean ± SEM.

3.3.5 Cell attachment assays

To determine if ghrelin had an effect on cell adhesion, a process which is required by ovarian cancer cells to allow them to attach to the peritoneum and to grow at this distant site [527, 528], we performed attachment assays using a range of extracellular matrix proteins. The SKOV3 and OV-MZ-6 ovarian cancer cell lines and hOSE 17.1 normal-derived cell line were cultured in the presence and absence of acylated ghrelin (0, 10 and 100 nM) and then assessed for their ability to adhere to the extracellular matrix proteins, fibronectin, vitronectin, collagen I and collagen IV (Fig. 3.7). A statistically significant mean 24.7 +/- 10.0 % decrease in adhesion to collagen IV was observed with the 10 nM ghrelin treatment in the SKOV3 ovarian cancer cell line (Fig. 3.7) ($P < 0.05$). No significant changes were seen with any of the other matrix molecules, cell lines or concentrations of ghrelin tested.

3.3.6 Ghrelin signalling assays

Ghrelin is known to stimulate activation of the ERK1/2 pathway [169, 178, 180, 183] and a number of studies have shown that it mediates the effects of ghrelin on cell proliferation [25, 40, 57, 168-172]. It has been previously demonstrated that ovarian cell lines have a low level of basal ERK1/2 expression [529], and therefore, we investigated the activation of ERK1/2 by ghrelin. In the SKOV3 cell line, 100 nM ghrelin treatment resulted in a statistically significant decrease (0.58 fold +/- 0.23) in ERK1/2 phosphorylation compared to the untreated control ($P < 0.05$) (Fig. 3.8). In the OV-MZ-6 cell line, ghrelin treatment caused a small, non-statistically significant increase in ERK1/2 phosphorylation at lower concentrations (0.1, 1 and 10 nM) and with 100 and 1000nM ghrelin treatments, decreased phosphorylation of ERK1/2 was observed compared to the untreated control, but this was not statistically significant. A dose-dependent increase in ERK1/2 phosphorylation in response to ghrelin treatment has been described in other studies [40, 169]

3.4 Discussion

We have demonstrated for the first time that ghrelin is expressed at the mRNA level in human ovarian cancer cell lines and in ovarian cancer specimens. Ghrelin treatment stimulates cell migration, but not proliferation, or invasion in the SKOV3 and OV-MZ-6 serous ovarian cancer cell lines. Ghrelin may also influence the adhesion of SKOV3 ovarian cancer cells to collagen IV, a key extracellular matrix

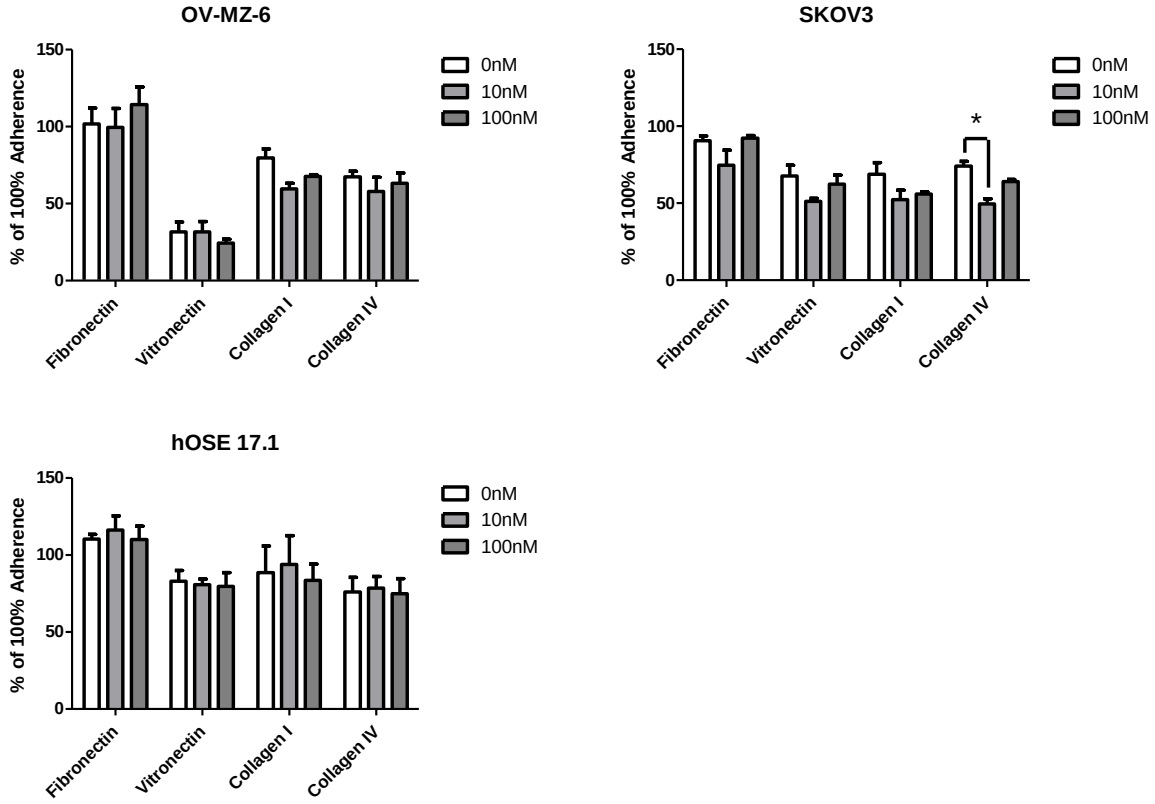

Figure 3.7 Attachment assays for SKOV3 and OV-MZ-6 ovarian cancer cell lines and the hOSE 17.1 normal ovarian epithelial cell line in response to ghrelin treatment. Attachment was measured using the CyQuant assay as a direct representation of the number of cells attached. Results are shown as a mean percentage (minus background) of the total number of cells that could possibly adhere to the uncoated 100% adherence plate with the aid of serum +/- SEM. *P <0.05, One way ANOVA and Tukey's *post hoc* test, compared to 0 nM control. n = 3 repeats with 3 replicates per independent experiment.

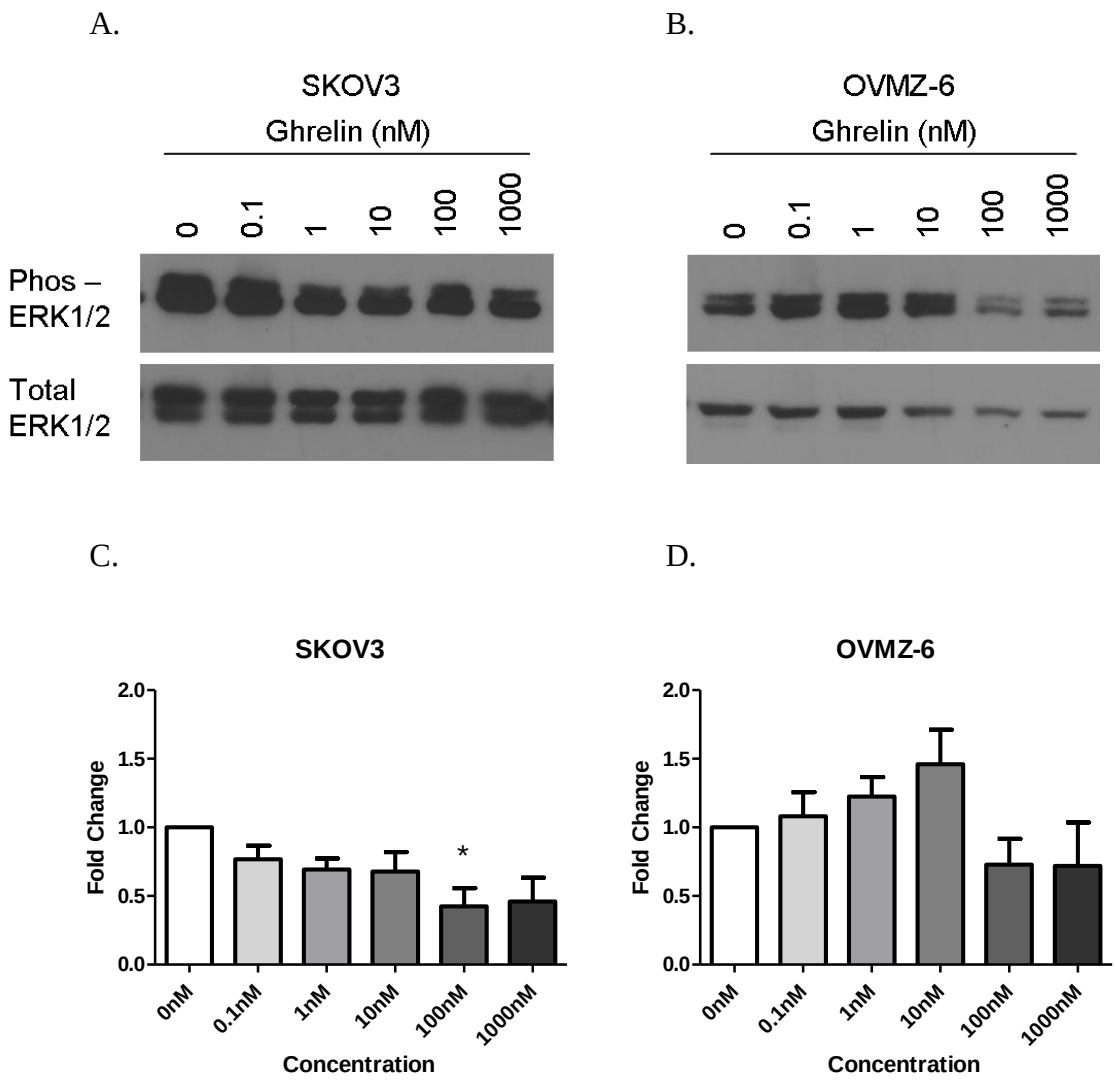

Figure 3.8 Western immunoblot analysis of ERK1/2 phosphorylation after ghrelin treatment in the SKOV3 and OV-MZ-6 ovarian cancer cell lines. Serum-starved cells were treated with a range of ghrelin concentrations (0, 0.1, 1, 10,100, and 1000 nM) for 15 min in the SKOV3 cell line and for 30 mins in the OV-MZ-6 cell line (determined as the peak ERK1/2 signalling time points for these cell lines). Representative Western immunoblot of triplicate experiments showing cell lysate analysed using anti-phosphorylated ERK1/2 and anti-total ERK1/2 antibodies in the (A) SKOV3 and (B) OV-MZ-6 ovarian cancer cell lines. Densitometry was performed to quantify the changes in protein expression with different ghrelin treatments in the (C) SKOV3 and (D) OV-MZ-6 ovarian cancer cell lines. The densitometry data are a mean of triplicate experiments (n = 3) (mean +/- SEM), corrected for total ERK1/2 present in each protein extract. *P <0.05 compared to 0 nM control using one way ANOVA and Tukey's *post hoc* test.

protein. Ghrelin may, therefore, play a role in ovarian cancer progression.

We have demonstrated that ovarian cancer cell lines and tissues express ghrelin mRNA, although expression was not correlated with the clinical stage of the specimens examined. It should be noted, however, that in this study the fold changes in expression are compared to normal ovarian samples. The normal tissue samples used are derived from the whole ovary and may contain little, if any ovarian surface epithelium, from which most ovarian cancers are derived. We have previously demonstrated the expression of ghrelin protein in normal ovarian specimens using immunohistochemistry [155]. In a recent semi-quantitative immunohistochemical study of serous ovarian tumours, ghrelin expression was increased in malignant tissue (n = 20) compared to benign samples (n = 20) [380]. In a small study, patients with benign ovarian tumours (31 patients) and ovarian cancer (22 patients) had higher blood levels of acylated ghrelin compared to a control group of women (n = 32), while the levels of total ghrelin were similar [114]. These authors concluded that elevated ghrelin plasma levels could indicate that ghrelin plays a role in the progression of ovarian cancer [114], although larger studies are clearly required to confirm these findings. As circulating ghrelin levels are strongly inversely correlated to body mass index (BMI), and ghrelin levels are elevated in cancer-related cachexia [530-532], ghrelin levels should be corrected for BMI. It is possible that ghrelin may be secreted from the ovarian tumours themselves.

GHSR1a mRNA was not found to be expressed in normal and cancerous ovarian cell lines and tissues, however, GHSR1b is expressed in the cell lines tested. Previous studies showed that GHSR1a protein could be detected in oocytes, as well as in somatic follicular cells, luteal cells from young, mature, old, and regressing corpora lutea, and interstitial hilus cells and strongly in the granulosa and thecal layers of healthy antral follicles of the normal ovary [155]. Expression has also been demonstrated in the ovarian surface epithelium and Műllerian duct derivatives, as well as in benign serous tumours resembling Fallopian tube epithelium and highly differentiated serous cystadenocarcinomas. It was not expressed in other ovarian neoplasms, such as mucinous cystadenomas, cystadenocarcinomas, endometrioid tumours, clear cell carcinomas, or Brenner's tumours [45]. In this study, GHSR1a was also not expressed in high grade serous cystadenocarcinoma, and this lack of

expression was also observed for the ovarian cancer cell lines used in this study which are both derived from high grade serous ovarian cancers. These previous studies demonstrated GHSR1a expression at a protein level using immunohistochemistry, whereas in this study expression was examined at an mRNA level and this may account for the differences in results. It is possible, but unlikely that GHSR1a mRNA levels were too low to detect in our studies. Alternative GHSR1a isoforms may be expressed by ovarian tissue, or the antibodies used in our previous studies may be non-specific, or detect related receptors [533].

Ovarian cancer and ovarian cancer cell lines also express GOAT (*MBOAT4*) mRNA, which encodes the enzyme that octanoylates ghrelin [534], and this indicates that ovarian cancer cells may be able to produce octanoylated (acylated) ghrelin, which could then act as a local factor on the cancer cells. We have hypothesized that locally produced ghrelin in tumours may play a more important role in cancer progression than circulating ghrelin [15]. In order to investigate the role of autocrine/paracrine ghrelin in ovarian cancer, tissue levels of octanoylated ghrelin should be measured to determine if octanoylated ghrelin is secreted from ovarian cancer cell lines after cells are treated with octanoic acid. Octanoic acid treatment is thought to be required for cells to produce octanoylated ghrelin in culture [122]. The expression of ghrelin and GOAT needs to be confirmed at the protein level in ovarian cell lines using a number of techniques including immunohistochemistry, Western immunoblotting, ELISA or mass spectrometry.

In this chapter it was demonstrated that ghrelin treatment increases the rate of cell migration in Transwell assays in the OV-MZ-6 and SKOV3 ovarian cancer cell lines, but not in the hOSE 17.1 normal-ovary derived cell line. While there have been a number of reports demonstrating that ghrelin treatment increases cell proliferation in normal and cancer derived cell lines [17, 25, 39, 47-51, 54, 57, 132, 168, 169, 175, 178, 189, 205, 319, 322, 395, 403, 404, 406, 409, 414, 509, 535-544], only a few studies have demonstrated that ghrelin can influence cell migration [16, 48, 54, 174]. Cell migration involves the accumulation of actin filaments at the leading edge of the cell producing a physical force that extends the membrane in the direction of the motility [423]. This protruding leading edge then adheres to the substratum, allowing the retraction of the rest of the cell and de-adhesion of the trailing end of the cell,

producing a crawling like motion [423-425]. Studies using phalloidin (an actin stain) have shown increased cellular staining in the presence of ghrelin in rat hippocampal slice cultures [426, 545]. In human T cells, human astrocytoma cell lines and rat hippocampal slice cultures, ghrelin stimulates actin polymerization [16, 267, 426]. The increased phalloidin-binding to F-actin in the rat hippocampal slice cultures was stemmed by the use of a ghrelin receptor antagonist [426]. The examination of astrocytoma Dixit *et.al.* demonstrated increased motility with increased actin polymerisation and membrane ruffling on cells with ghrelin treatment [16]. Increases in intracellular calcium mobilization, PKC activation, MMP2 activity and co-localization of the small GTPase, Rac1, with GHSR on the leading edge of the cells and membrane ruffling on cells were also observed [16]. Confocal microscopy of the ovarian cell lines could be performed to investigate whether the membrane ruffling observed in the astrocytoma cell lines [16] and accumulation of actin [16, 267, 426] also occurs in ovarian cancer cells. In the astrocytoma cell lines, increased migration was shown to be dependent on GHSR1a by disruption of the endogenous ghrelin-GHSR pathway by siRNA [16]. Collectively, all of these studies suggest that ghrelin may influence the remodelling of cytoskeleton proteins, and actin specifically, in the process of migration. The GHSR1a is unlikely to be expressed, or play a role in cell migration in ovarian cancer cell lines, however.

Ghrelin has actions in a number of cell types that do not express the ghrelin receptor, indicating that an unknown ghrelin receptor type exists [157, 178, 195, 196, 206, 207, 248, 394, 546, 547]. Despite previously demonstrating that GHSR1a mRNA is expressed in prostate, endometrial and breast cancer [17, 39, 49], neither the ovarian cell lines examined in our study, nor the 48 clinical specimens tested, expressed GHSR1a mRNA. Therefore, ghrelin must stimulate cell migration in ovarian cancer cell lines by signalling through the hypothesized alternative ghrelin receptor [548]. GHSR does appear to play a role in migration and invasion in the SW-48 and RKO colorectal cancer cell lines, as GHSR antagonists inhibit these effects [48]. GHSR1a-independent stimulation of cell migration may exist in malignant colorectal cells, however, GHSR1a expression is dramatically lost with malignancy and the effects of ghrelin on proliferation, migration and invasion must be mediated through the alternative ghrelin receptor [48].

Differences in receptor expression between cell types and cell lines could explain the different responses to ghrelin treatment. This could be investigated further in ovarian cancer cell lines by expressing GHSR1a and investigating the effects of ghrelin on proliferation and migration for any changes in response. The alternative receptor is thought to bind both ghrelin and desacyl ghrelin, as both forms of ghrelin are functional and can differentially regulate processes such as proliferation [168, 175, 422] and apoptosis [175, 177, 178, 415]. As the alternative receptor can bind both forms of ghrelin, it is may also be susceptible to GHSR1a antagonists. The GHSR1a inverse agonist, D-Lys3-GHRP-6, has been shown to inhibit desacyl ghrelin-stimulated growth of HECa10 murine endothelial cells [549]. D-Lys3-GHRP-6 is also non-specific, as it also binds the CCR5 chemokine receptor [550]. This could explain why there was an inhibition of the ghrelin-stimulated migration in the study of colorectal cell lines treated with the ghrelin antagonist when it was unlikely the response was being mediated through GHSR1a [48].

Ghrelin treatment did not alter cell invasion through Matrigel in the SKOV3, OV-MZ-6 or hOSE 17.1 ovarian cell lines compared to controls in Transwell assays, and ghrelin may, therefore, not play a role in increasing cell invasion in ovarian cancer. In pancreatic and colon cancer cell lines, ghrelin has been shown to increase invasion through Matrigel in Boyden chamber assays [48, 54]. Conversely, in patients with oral squamous cell carcinoma (OSCC), decreased or absent expression of ghrelin in oral biopsy specimens was correlated with increased invasiveness of the disease [24].

We have previously demonstrated that ghrelin stimulates cell proliferation in prostate, breast and endometrial cell lines [17, 40, 49], and other studies have demonstrated that ghrelin stimulates cell proliferation in a wide range of normal and cancer cell lines [17, 25, 39, 47-51, 54, 57, 132, 168, 169, 175, 178, 189, 205, 319, 322, 395, 403, 404, 406, 409, 414, 535-544]. In contrast, ghrelin treatment did not stimulate cell proliferation in the SKOV3 and OV-MZ-6 cancer cell lines, or the normal ovarian cell line, hOSE 17.1, in this study. Ghrelin may have different actions in different cell types and some studies have also demonstrated that ghrelin can reduce proliferation, or have no effect on cell proliferation in other cell lines [18, 28, 44]. Given that ghrelin has a very short half life, it is unlikely that proliferation is being masked by endogenous ghrelin in the culture medium [145, 146]. To confirm

this, however, knockdown studies should be performed to confirm that endogenous expression of ghrelin is not interfering with this assay and to investigate the role of endogenous ghrelin further.

In addition to migration and invasion, epithelial ovarian cancer cell metastasis requires the attachment of cells to the mesothelium and invasion into the mesothelium of the peritoneum, which lines the peritoneal cavity [527, 551]. This attachment is aided by the fact that mesothelial cells express a number of ECM molecules, including fibronectin, vitronectin and collagen I on their cell surface [552-554]. They do not express collagen IV, which is usually a component of the basement membrane. To investigate if ghrelin may influence adhesion, attachment assays were performed using wells coated with ECM components (fibronectin, vitronectin, collagen I and collagen IV) and cells were treated with different concentrations of ghrelin. In this study, ghrelin did not alter attachment to fibronectin, vitronectin or collagen I, but a significant decrease in attachment to collagen IV was seen in response to 10 nM ghrelin in the SKOV3 ovarian cancer cell line, but not in the OV-MZ-6, or hOSE 17.1 cell lines. Although collagen IV is not expressed by the mesothelial cells themselves, this may aid attachment to the underlying basement membrane, which is also required for cells to invade through the peritoneum.

Although there have been no studies in cancer, other studies have demonstrated that the ghrelin axis may play a role in cell adhesion in immune cells [268]. In atherosclerosis, ghrelin increased the adhesion of THP-1 monocytes to an endothelial cell line, but decreased adhesion when cells were also treated with tumour necrosis factor-alpha (TNF-α), indicating that ghrelin may play a different role during inflammation [555]. In THP-1 monocytes, ghrelin inhibited the expression of the cell adhesion molecules, vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1) [555]. This contrasts with another study in monocytes which showed an increase in ICAM-1 (VCAM-1 expression inter-cellular adhesion molecule 1) expression with ghrelin treatment [556]. Although increases in the expression of adhesion molecules were seen in this study, ghrelin did not alter monocyte adhesion itself, but ghrelin may contribute to atherogenesis [556]. Ghrelin has also been demonstrated to reduce the TNF-α stimulated adhesion of the U937

human lymphoma cell line to human umbilical vein endothelial cells (HUVEC) [268].

Dysregulation of growth factor expression and signalling pathways in tumour cells often results in autocrine/paracrine effects that ultimately promote cancer cell processes, such as proliferation and migration [557-559]. Characterising the effects of growth factors in cancer cells may, therefore, provide important insight into their function in normal physiology and disease. A number of studies have implicated ghrelin as an autocrine/paracrine growth factor in human tumours, including breast cancer [17], prostate cancer [39, 40], colorectal cancer [48], thyroid cancer [43, 560], lung cancer [27], leukemia [51], hepatoma [25], astrocytoma [16], and pituitary adenoma [57]. Activation of the ERK1/2 MAPK pathway is one of the most widely studied ghrelin signalling pathways which mediates a number of cell processes including cell proliferation [25, 38-40, 57] and apoptosis [40] in cancer and in normal cell lines [168-172, 175-180]. Ghrelin acts through the ERK1/2 pathway to stimulate cell proliferation in a number of cell lines, including prostate cancer cell lines [40], 3T3-L1 adipocytes [404], human adrenal zona granulosa cells [542] and the rat GH3 cell line [57]. In all of these cell lines this action appears to be the downstream effect of activation of GHSR1a by ghrelin. Ghrelin also stimulates cell proliferation through the ERK1/2 pathway in the absence of GHSR1a in human osteoblasts, presumably through the alternative ghrelin receptor [168]. Ghrelin signalling through ERK1/2 also protects a number cell types against apoptosis, including oligodendrocytes, endothelial cells, beta pancreatic islet HIT-T15 cells and porcine ovarian follicular cells [179, 183, 509, 561].

While ghrelin has previously been shown to increase ERK1/2 activation in the SKOV3 cell line at high concentrations (1 μmol/L) [529], the effect of ghrelin treatment on ERK1/2 activation has not been investigated in ovarian cell lines at physiologically relevant concentrations. In contrast, in this study we have demonstrated by Western blot analysis, that ghrelin does not activate ERK1/2 in ovarian cancer cell lines. In fact the 100nM ghrelin treatment down-regulated ERK1/2 phosphorylation in the SKOV3 ovarian cancer cell line. A similar effect was also seen with the OV-MZ-6 cancer cell line, although this was not statistically significant. This contrasts with many other studies in other cancer tissues which

demonstrate that ghrelin function is accompanied by increased ERK1/2 phosphorylation, often in a dose-dependent manner [39, 40, 57]. These studies, however, demonstrated proliferative effects with ghrelin treatment rather than cell migration. Regulation of proliferation via the ERK1/2 signalling pathway is a well-documented effect of ghrelin [25, 40, 57, 168-172] and is often accompanied by the expression of GHSR1a [25, 39, 40, 57, 171, 172]. Additionally, blocking GHSR1a results in a down regulation of ERK1/2 signalling [171, 172]. This correlates with the results of this study, as the ovarian cancer cell lines did not activate ERK1/2 or expressed GHSR1a and had no effect on proliferation.

In studies of microvascular endothelial cells, migration has been shown to coincide with increased ERK1/2 phosphorylation [171, 172, 174]. In these studies, however, it was not determined whether inhibition of the ERK1/2 signalling affected cell migration, but it did decrease angiogenesis [171, 172, 174]. Ghrelin did stimulate migration in the ovarian cancer cell line, however, the lack of stimulation of the ERK1/2 phosphorylation by ghrelin in these cell line suggest that it is not involved in this response.

A number of studies have investigated the pathways activated by ghrelin-stimulated cell migration. A study on glioma cells demonstrated that ghrelin-stimulated cell migration was primarily mediated through GHSR1a, and through calmodulin-dependent protein kinase II (CaMKII), AMP-activated protein kinase (AMPK), and nuclear factor-kappa B (NF-κB) pathway [55]. Pancreatic adenocarcinoma cell lines, PANC1, MIAPaCa2, BxPC3 and Capan2, displayed an increase in Akt activation and an increase in cell migration after ghrelin treatment [54]. Ghrelin-stimulated migration in these pancreatic adenocarcinoma cell lines was inhibited by the GHSR1a antagonist, D-Lys3-GHRP-6, and the PI3-K inhibitor, Wortmannin [54]. Ghrelin-stimulated astrocytoma motility is mediated through GHSR1a signalling, resulting in an increase in intracellular calcium mobilization, PKC activation, actin polymerization and increased MMP2 activity [16]. Although ovarian cancer cell lines do not express the GHSR1a, these signalling pathways could be activated by the alternative ghrelin receptor and also play a role in ghrelin-stimulated cell migration. Investigation of these other pathways in the ovarian cell lines using Western blots analysis, or ELISA, and assaying phosphorylation of signalling molecules is required

to determine if they play a role in the ghrelin-stimulated migration seen in the ovarian cancer cell lines. It is very likely that ghrelin-stimulated cell migration is regulated by a number of different signalling pathways.

Ovarian cancer is the most fatal gynaecological cancer and epithelial ovarian cancers are highly metastatic [562]. Ovarian cancers are more frequently diagnosed at a late stage in the disease and this contributes to a poor prognosis. Markers for the early detection of ovarian cancer and new therapeutic approaches are urgently required. In one small study, plasma ghrelin levels have been shown to be elevated in ovarian cancer patients [114] and it is feasible that elevated plasma ghrelin may promote cell migration and cancer progression in ovarian cancer patients. As ghrelin is expressed by ovarian cancer cells, autocrine or paracrine ghrelin may also play a role in ovarian cancer progression and knockdown studies in ovarian cancer cell lines would be helpful in elucidating the mechanism of action of ghrelin and confirming the results seen in this chapter.

In conclusion, our present study demonstrates that normal ovarian surface epithelial cells and ovarian cancer cells and cell lines express ghrelin mRNA, but not GHSR1a. It would be useful to quantify the level of ghrelin expression in ovarian cancer tissues themselves by Western blot analysis, ELISA or mass spectrometry. Ghrelin was demonstrated to promote cell migration, but not proliferation in cultured ovarian cancer cell lines. Antagonizing the effects of ghrelin in ovarian cancer could provide a new additional therapeutic approach which could reduce the rate of ovarian cancer progression, by inhibiting cell migration.

CHAPTER 4

Expression and function of obestatin in ovarian cancer cell lines

4.1 Introduction

Obestatin is a 23 amino acid peptide hormone which is derived from the ghrelin preprohormone [6] and encoded by the ghrelin gene (*GHRL*). Ghrelin and obestatin, two distinct peptide hormones, are both cleaved from the 117 amino acid preproghrelin peptide [1, 6, 116]. Ghrelin potently stimulates appetite [1, 563] and obestatin was initially thought to have the opposite effect of ghrelin on appetite [7]. This study has proven controversial, however, and many studies have failed to replicate the effects of obestatin on feeding [435, 442, 452-454, 461, 475, 480, 481, 483-485, 490, 564, 565]. Although it was originally reported that GPR39, a G protein coupled receptor in the ghrelin receptor family, was the obestatin receptor, it is now clear that GPR39 is a zinc receptor and not the obestatin receptor [7]. The identity of the obestatin receptor is currently unknown [7]. Despite these controversies, however, it is clear that obestatin is a multifunctional hormone, and it may play a role in cancer progression [4, 7].

Ghrelin appears to play an autocrine/paracrine role in processes related to cancer progression [15], including the stimulation of cell proliferation [17, 25, 39, 40, 49, 51] and cell migration [16, 48, 54] and the inhibition of apoptosis [47, 49, 52]. The ghrelin preprohormone derived peptide, obestatin, may also play a stimulatory role in cancer-related processes. Obestatin stimulates cell proliferation in some cell lines, including retinal pigment epithelial cells [10] and the KATO-III gastric cancer cell line [11], but inhibits proliferation in other cell lines, including the TT-human medullary thyroid carcinoma cell line and the BON-1 pancreatic endocrine tumour cell line [56]. The preproghrelin peptide is transcribed in the ovary (Chapter 3.3.1) and using immunohistochemistry, it has been shown that the ghrelin receptor, GHSR1a, is expressed in ovarian cancer [45]. Obestatin expression has been demonstrated immunohistochemically in benign and malignant serous tumours [380]. Although obestatin levels were not elevated in malignant tumours, compared to benign tumours, the level of expression of obestatin in normal ovarian surface epithelium was unclear in this study [380]. Obestatin expression did not appear to correspond to tumour grade [380]. Additionally, octanoylated ghrelin and obestatin plasma levels are elevated in benign ovarian tumours and in ovarian cancer patients [114], however, it is not clear if the ovarian tumours are the source of the elevated obestatin levels.

Ovarian cancer is the leading cause of death for all gynaecological malignancies [566] and as there are few symptoms, ovarian cancer is difficult to diagnose at a low tumour grade [66]. As a result, two thirds of ovarian cancer patients are not diagnosed until the late stages of cancer development, (at either stage III or IV), resulting in a poor prognosis [61]. Early diagnosis leads to better patient outcomes, and better biomarkers for the early detection of ovarian cancer are urgently required.

In this study it was demonstrated that the coding region for obestatin is expressed in ovarian cancer cell lines and in ovarian tumour clinical specimens. We hypothesised that obestatin could be an autocrine/paracrine growth factor in ovarian cancer, or circulating obestatin may stimulate ovarian cancer cell progression. In this study, we have demonstrated that obestatin treatment stimulates cell migration in the SKOV3 and OV-MZ-6 ovarian cancer cell lines and increases cell invasion in the OV-MZ-6 ovarian cancer cell line and hOSE normal ovarian surface epithelium cell line, compared to the untreated control.

4.2 Methods

4.2.1 Statement of contribution

The practical work described in this Chapter was performed in collaboration between the candidate, Carina Walpole, and Dr Inge Seim. Dr Inge Seim performed the quantitative real time RT-PCR of the ovarian cancer tissues (Results 4.3.1) in consultation with the candidate, while the candidate performed all other experiments.

4.2.2 Cell lines and cell culture

In this study, assays were performed using the SKOV3 [511] and OV-MZ-6 human epithelial ovarian serous adenocarcinoma cell lines [510] and the hOSE 17.1 normal human ovarian surface epithelium-derived cell line [512]. The SKOV3, OV-MZ-6 and hOSE 17.1 cell lines used in this study were propagated as described in the General Methods, Chapter 2.1.

4.2.3 Tissue immunohistochemistry

In order to determine if there is a relationship between cancer grade and the level of expression of obestatin, a semi-quantitative, immunohistochemical approach was

used [40]. Twenty ovarian tissue slides, consisting of ovarian cancer samples of varying grade, benign samples and normal ovarian tissue were available through a collaboration with Dr Obermair (Queensland Centre for Gynaecological Cancer, Royal Brisbane and Women's Hospital, Brisbane). Tissue sections were formaldehyde-fixed, paraffin embedded histopathology ovarian cancer specimens and normal ovarian tissue. Tissue sections were microwaved in citric acid buffer for 10 minutes (pH 6) to enhance antigen retrieval. Tissue sections were incubated with an anti-obestatin antibody raised in rabbit (Phoenix pharmaceuticals) and with antibodies raised in rabbit (Institute of Medical and Veterinary Science) against the full length obestatin peptide (Mimotopes) and affinity purified. Primary antibody was diluted 1/200 in 0.01 M PBS with 1% bovine serum albumin (BSA) (Sigma, St Louis, MO, USA), and incubated with tissue sections overnight at 4ºC. After washing in 0.3% Triton X-100 (Merck Millipore, Billerica, MA, USA) in PBS, sections were incubated in goat anti-rabbit secondary antibody (DAKO Envision Plus Kit, Dako, Kyoto, Japan) for 30 min at room temperature. After washing, immunostaining was performed using a DAKO Envision Plus Kit using DAB as a chromogen (Dako), according to the manufacturer's instructions and tissues were counterstained using haematoxylin (Australian Chemical Reagents, Moorooka, Australia). Negative controls were performed with the omission of the primary antibody. While it was not possible to accurately compare samples quantitatively (due to different fixation conditions of tissue samples), staining was described in terms of: non-staining, low, medium and high intensities of staining.

4.2.4 Quantitative real-time RT-PCR of clinical ovarian cancer tissue specimens

Real-time RT-PCR, used to quantify expression levels of obestatin-coding (*GHRL* exon 3 spanning) mRNA transcripts, was performed using cDNA from normal ovary and from ovarian cancer patients using cancer tissue panels (OriGene TissueScan qPCR Ovarian Cancer panel II, OriGene, Rockville, MD) as described in Chapter 2.2. This array consists of pathologist-verified samples from eight non-tumour ovarian tissues and 40 different ovarian tumours, with a range of cancer stages.

4.2.5 Cell proliferation assays

WST-1 and CyQuant cell proliferation assays were performed as described (Chapter 2.4) using the SKOV3 and OV-MZ-6 ovarian cancer cell lines and the hOSE 17.1

normal cell line with a range of obestatin concentrations (0, 0.1, 1.0, 10, 100, 1000 nM) and no treatment controls (0 nM). Each treatment (and control) was performed using 16 replicate wells and each experiment was performed independently at least 3 times. Standard curves were performed to confirm a linear correlation between cell number and absorbance or fluorescence values.

4.2.6 Cell migration and invasion assays

Migration and invasion assays were performed using the SKOV3 and OV-MZ-6 ovarian cancer cell lines and the hOSE 17.1 normal-derived ovarian cell line using Transwell assays (as described in Chapter 2.5). For invasion assays, the upper surfaces of the inserts were coated with growth factor-reduced synthetic ECM Matrigel (BD Biosciences). Cells were seeded in the upper chamber of the Transwell in FCS-free growth medium with or without obestatin (0, 10 and 100 nM). Controls were performed to determine background staining using inserts without cells, containing medium only. For each experiment 3 replicates per treatment were performed and the experiments were repeated independently 3 times.

4.2.7 Attachment assays

To assess whether obestatin treatment had any effect on cellular attachment, the hOSE 17.1 normal ovarian cell line and OV-MZ-6 and SKOV3 ovarian cancer cell lines were treated with obestatin (0, 10 and 100 nM) and then tested for their ability to attach to the extracellular matrix (ECM) proteins fibronectin, vitronectin, collagen I and collagen IV. Attachment assays were performed as described in Chapter 2.6. The assay was performed in triplicate with 3 independent replicates of each ECM molecule per assay.

4.2.8 Statistics

Statistical analyses were undertaken as described in Chapter 2.8 using either one-way ANOVA, followed by Tukey's *post hoc* test (with $P < 0.05$ considered to be statistically significant) or a two-way ANOVA, followed by Bonferoni's *post hoc* analysis (with $P < 0.05$ being considered to be statistically significant).

4.3 Results

4.3.1 Obestatin mRNA and protein is expressed by ovarian cancer tissues

The expression of the obestatin-coding region of preproghrelin (*GHRL* exon 3-4) was examined by quantitative real-time RT-PCR using normal and ovarian cancer tissue from ovarian carcinoma patients (Fig. 4.1). Although levels of obestatin-containing transcripts varied in different ovarian cancer samples and were generally higher than normal ovarian tissue, there was no strong correlation between the cancer stages and obestatin mRNA expression. In a subset of tumours (≈ 6/40), however, expression was more than 10 fold the expression in normal tissue, and therefore, obestatin expression appears to be upregulated in a subset of ovarian cancers. Normal ovarian tissue in this study is likely to consist of a range of different cell types and little if any ovarian surface epithelium, the most common site from which tumours develop. The coding region for obestatin was expressed in the majority of the samples tested. mRNA expression of the ghrelin gene, including the coding region for obestatin, was demonstrated in the SKOV3 and OV-MZ-6 ovarian cancer cell lines and hOSE 17.1 normal ovarian cell line (Chapter 3.3.1, Fig 3.3).

We demonstrated that the obestatin peptide is also expressed in human ovarian cancers using immunohistochemistry. This antibody is raised against the obestatin peptide, and could also detect proghrelin and preproghrelin, (prior to processing of the prepropeptide into the ghrelin and obestatin peptides). The ovarian surface epithelium in normal ovarian tissue demonstrated very low or absent levels of obestatin immunoreactivity (Figure 4.2 A). Positive granular obestatin immunoreactivity was demonstrated in the cytoplasm of endometrioid (n = 2) and papillary serous ovarian tumours (n = 4) (Figure 4.2 B, C, D). Staining was observed throughout the cytoplasm, or concentrated in the apical region of the cell. Nests of epithelial cells in a benign Brenner's tumour show cytoplasmic obestatin staining, and less intense staining was observed in the surrounding stroma (Fig 4.2 E). In a case of salphingitis, strong obestatin immunoreactivity was observed in the cytoplasm of inflammatory cells (Fig 4.2 F). Luteal cells of the corpus luteum of the normal ovary also expressed granular cytoplasmic obestatin immunoreactivity (Fig 4.2 G). Negative controls, where the primary antibody was omitted, demonstrated no specific staining (Fig 4.2 H).

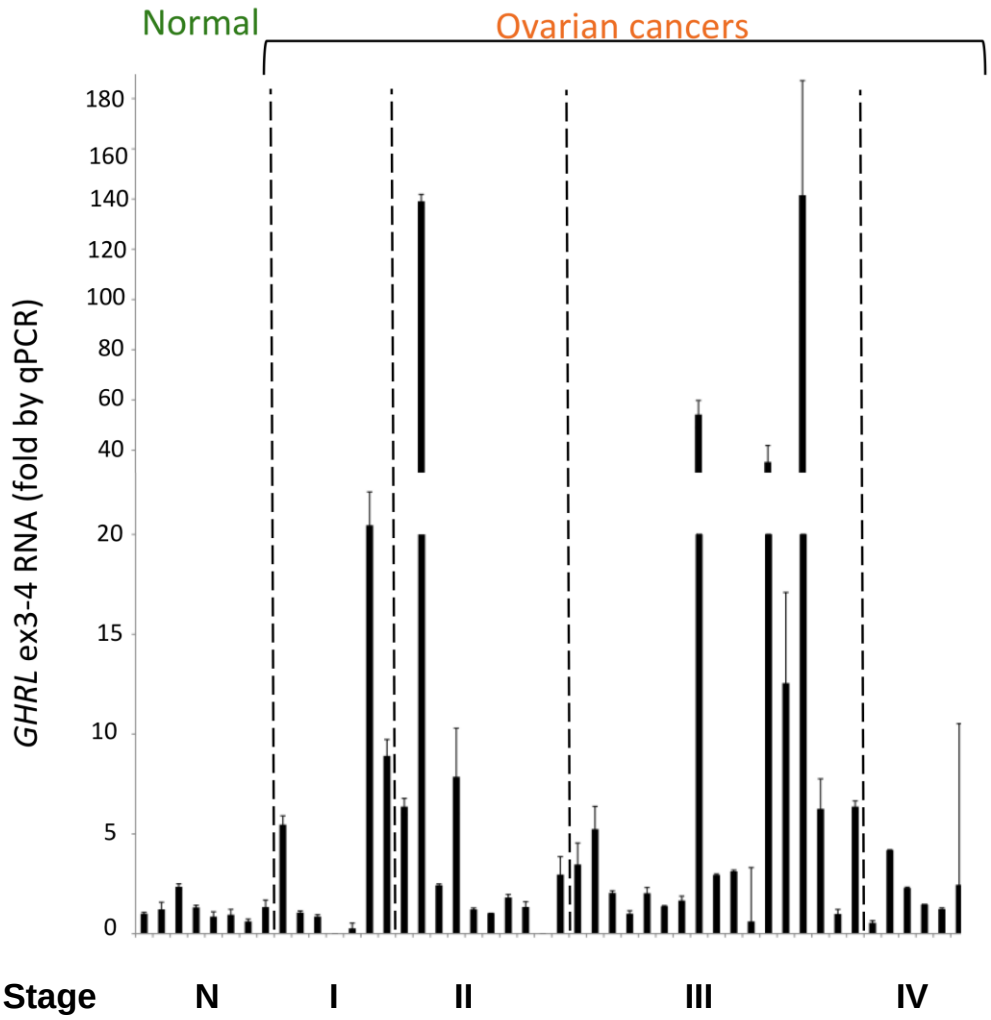

Figure 4.1 Quantitative RT-PCR demonstrating level of expression of the obestatin-coding region of the ghrelin prepropeptide mRNA (*GHRL* exon 3-4) in normal ovary and stage I to IV ovarian cancer. Quantitative real-time RT-PCR was carried out using an Origene TissueScan ovarian cancer panel. Negative controls were performed where template was substituted with water. Data were normalised to β-actin and are represented as fold changes, quantified as $2^{-(\Delta Ct\ sample-\Delta Ct\ control)}$, relative to expression of transcripts in a normal (N) ovarian tissue sample (1.0). Bars indicate mean +/- SEM (n = 2).

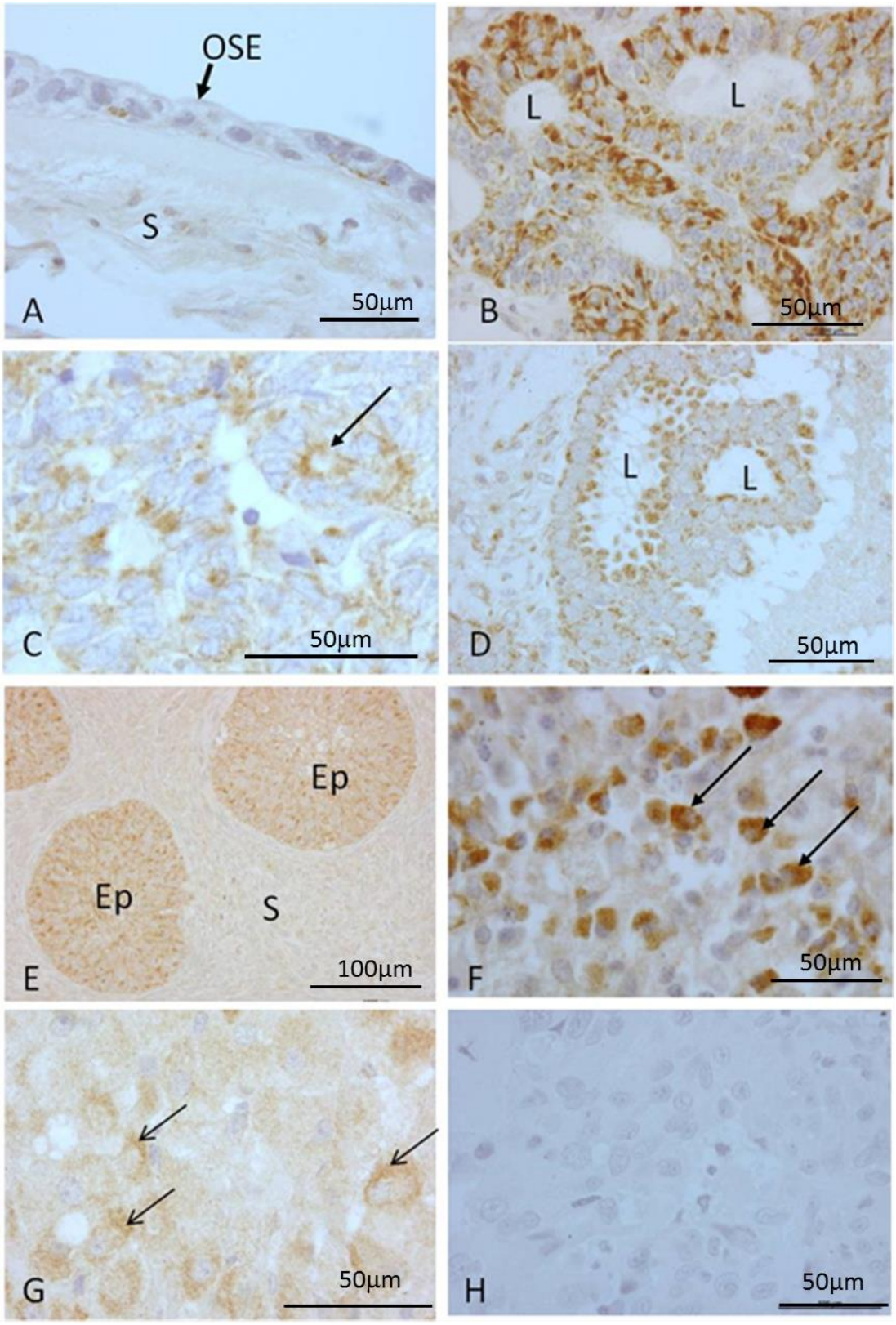
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Figure 4.2 Obestatin-like immunoreactivity is expressed in ovarian cancer and normal ovarian tissues.

Ovarian specimens showing obestatin immunoreactivity (brown staining). Immunohistochemistry was performed using clinical specimens using the Dako Envision plus kit with DAB as chromagen and nuclei were counterstained blue with haematoxylin. A. Normal ovarian surface epithelium (OSE) with very low levels of obestatin immunoreactivity and some immunoreactivity in the underlying stroma (S). B. Grade 3 ovarian serous adenocarcinoma (endometrioid) section showing strong obestatin immunoreactivity in the cytoplasm of epithelial cells and non-immunoreactive nuclei are counterstained blue. C. Obestatin immunoreactivity (brown) in serous ovarian cancer, with increased levels of staining in the apical regions of the glands. D. Papillary serous ovarian tumour demonstrating granular, obestatin-specific immunoreactivity in the epithelial cytoplasm and non-staining nuclei. E. Brenner's tumour showing obestatin immunostaining in the cytoplasm of epithelial cells (Ep) and moderate staining in the stroma. F. Inflammatory cells in a section of tissue from a case of salphingitis show moderate to high levels (arrows) of cytoplasmic obestatin expression. G. Normal corpus luteum of the ovary, showing granular perinuclear staining (arrows) in the luteal cells. The nuclei do not show obestatin immunoreactivity. H. Representative negative control showing serous ovarian carcinoma section where the primary antibody was omitted. L = glandular lumen.

4.3.2 Obestatin increases cell migration

To examine if obestatin stimulates cell migration in the SKOV3 and OV-MZ-6 serous adenocarcinoma ovarian cancer cell lines, Transwell migration assays (with 8 µm pores) were employed. There was a statistically significant increase in cell migration in response to 10 nM (fold increase of 1.74 +/- 0.30 SEM) and 100 nM obestatin (2.08 +/- 0.54 SEM) in the SKOV3 ovarian cancer cell line ($P < 0.05$) and increased in response to 10 nM (fold increase of 2.17 +/- 0.34 SEM, $P < 0.01$) and 100 nM obestatin (fold increase of 2.13 +/- 0.37 SEM, $P < 0.05$) in the OV-MZ-6 ovarian cancer cell lines (Fig. 4.3). Although cell migration was observed, there was no difference in cell migration in the hOSE 17.1 normal ovary-derived ovarian cell line in response to obestatin treatment compared to the non-treated controls (Fig. 4.3).

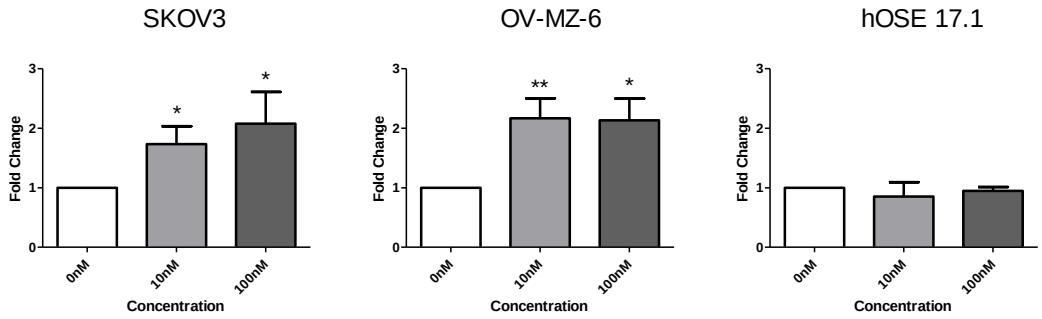

Figure 4.3 Obestatin treatment increases cell migration in the SKOV3 and OV-MZ-6 ovarian cancer cell lines compared to controls, but not in the hOSE 17.1 normal human ovary derived cell line in Transwell assays. Cells were seeded in Transwell inserts with 8 µm pores in growth medium with obestatin (10 and 100 nM) or controls (0 nM) and 10% FCS was added to the lower chamber as a chemoattractant. Migration was assessed by staining migrated cells with 1% crystal violet, and absorbance measured as a representation of the number of migrated cells. Graph represents mean fold change +/- SEM. *$P < 0.05$ and **$P < 0.01$ compared to 0 nM control using one way ANOVA and Tukey's *post hoc* comparisons. n = 3 repeat experiments with 3 replicates per experiment.

4.3.3 Obestatin does not stimulate proliferation in ovarian cancer cell lines

Obestatin has been shown to stimulate cell proliferation in some cancer and normal cell types [10-12, 465] and inhibit cell proliferation in others [13, 14] and we hypothesised that obestatin may be a growth factor in ovarian cancer (Fig. 4.4).

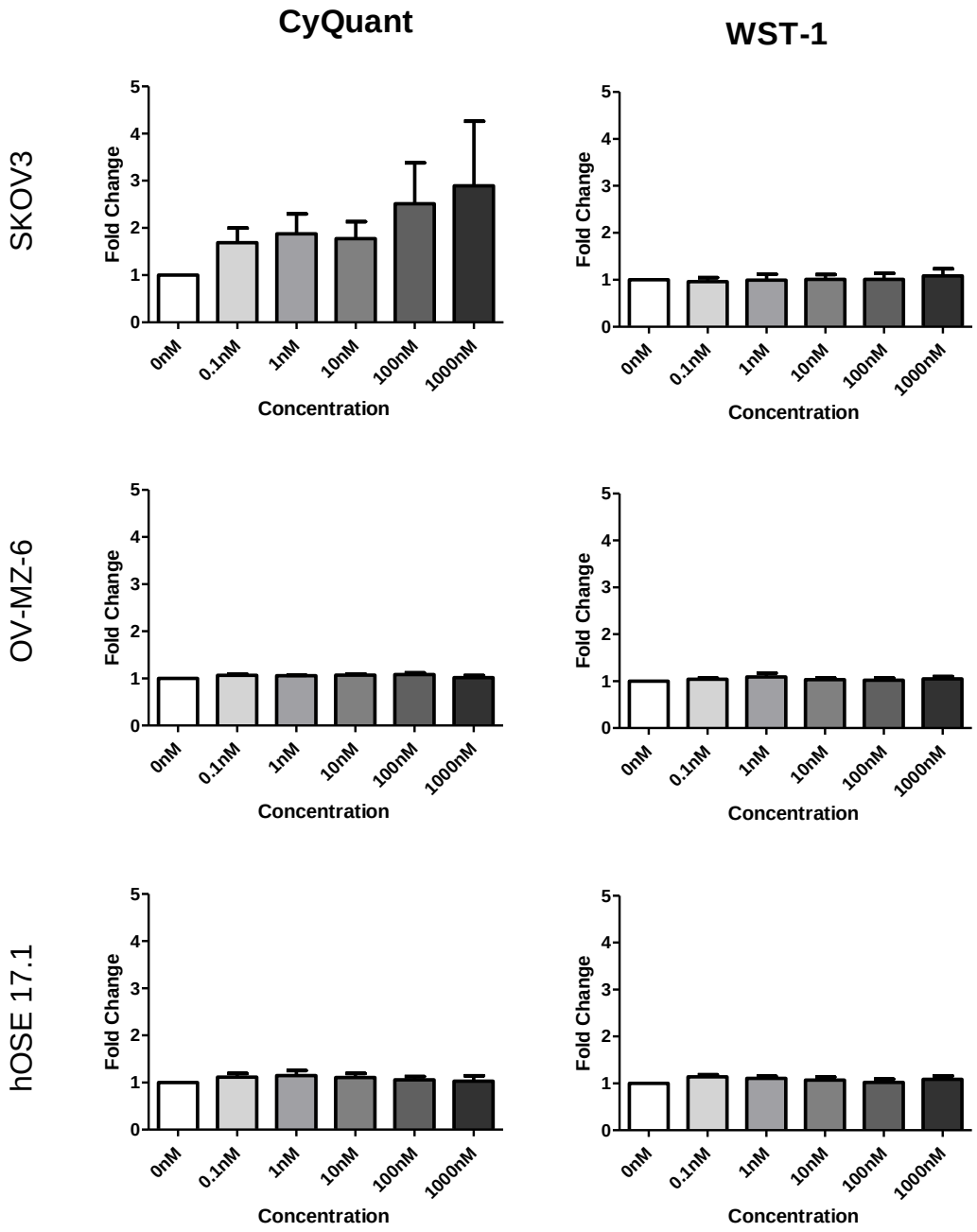

Figure 4.4 Obestatin treatment does not stimulate an increase in cell proliferation compared to controls in the SKOV3 or OV-MZ-6 ovarian cancer cell lines, or the normal ovarian cell line hOSE 17.1. Cell proliferation was determined using the CyQuant (DNA based) and Wst-1 (metabolic based) cell proliferation assays after treatment with obestatin for 72 h. Data is shown as mean proliferation +/- SEM (n = 3). Data was analysed using one way ANOVA. No statistically significant differences were observed.

Although all of the cell lines in the study were observed to proliferate over the 72 hours time period, treatment with a range of concentrations of obestatin (0.1-1000 nM) did not stimulate a statistically significant change in cell proliferation compared to the untreated controls (0 nM) in the SKOV3 or OV-MZ-6 ovarian cancer cell lines, or in the hOSE 17.1 normal ovary derived cell line in this study (Fig. 4.4). Cell proliferation was determined using a CyQuant assay to measure changes in DNA content and a WST-1 assay to measure changes in cell metabolism. As obestatin did not influence cell proliferation, it is unlikely that proliferation and increased cell number influenced cell migration in the SKOV3 and OV-MZ-6 cell lines.

4.3.4 Cell invasion assays

As it was demonstrated that treatment with obestatin increases migration in ovarian cancer cell lines, Transwell assays were performed to determine if obestatin could also alter the rate of cell invasion through Matrigel extracellular matrix (Fig. 4.5). Obestatin treatment increased invasion with both 10 nM and 100 nM treatments in the hOSE 17.1 cells (fold change of 1.40 +/- 0.04 SEM and 1.55 +/- 0.05 SEM respectively, P <0.01) and with 100 nM treatment in the OV-MZ-6 cancer cell line

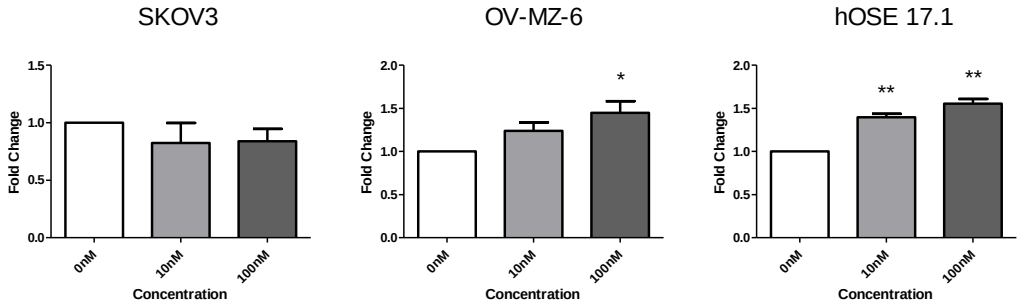

Figure 4.5 Invasion assays with ovarian cancer cell lines SKOV3 and OV-MZ-6 and normal ovary derived hOSE 17.1 cell line in response to obestatin treatment using Transwell assays and Matrigel. Obestatin treatment significantly increased cell invasion in the OV-MZ-6 ovarian cancer cell line and hOSE 17.1 normal cell line, but had no statistically significant effect on invasion in the SKOV3 ovarian cancer cell line. Transwell assays were performed over 48 h (SKOV3 and OV-MZ-6) or 72 h (hOSE 17.1). Mean fold change +/- SEM. *P <0.05 and **P <0.01 compared to 0 nM control using one way ANOVA and Tukey's *post hoc* comparisons. n = 3 repeat experiments with 3 replicates per experiment.

(fold change of 1.45 +/- 0.13 SEM, P <0.05). Interestingly, the SKOV3 ovarian cancer cell line did not show increased invasive potential following obestatin treatment (Fig. 4.5).

4.3.5 Cell attachment assays

As ovarian cancer cells must attach to the mesothelium (which lines the peritoneal cavity) in order to metastasise [527], assays were performed to determine the effect of obestatin treatment on cell attachment. Cells were treated with obestatin (or control), and its effect on attachment to a number of ECM components that are important to ovarian cancer was investigated. While collagen IV is not expressed by mesothelial cells it is a normal component of the basement membrane [552-554]. The SKOV3 and OV-MZ-6 ovarian cancer cell lines and the hOSE 17.1 normal cell line were cultured in the presence and absence of obestatin (10 and 100 nM) and assessed for their ability to adhere to fibronectin, vitronectin, collagen I and collagen IV. No significant difference in the number of cells attached (measured using CyQuant) was seen between the cell lines in response to the obestatin treatments (10 and 100nM) compared to the untreated controls (Fig 4.6).

4.4 Discussion

These studies have demonstrated that obestatin is expressed in ovarian cancer tissues at the mRNA and peptide level and that exogenous obestatin stimulates cell migration in the OV-MZ-6 and SKOV3 serous ovarian cancer cell lines, but not in the hOSE 17.1 normal ovarian surface epithelial cell line. Obestatin treatment also increased the rate of cell invasion through Matrigel in the OV-MZ-6 ovarian cancer cell line and the hOSE 17.1 normal-ovary derived cell line, but not the SKOV3 cancer cell line in Transwell invasion assays. This is the first demonstration that obestatin can stimulate cell migration in cancer cell lines. These data suggest that obestatin, like ghrelin (which also stimulated migration, but not cell invasion) (Chapter 3), may be an autocrine/paracrine factor in the ovary that stimulates cancer progression.

In the current study it was shown, for the first time that mRNA for the obestatin-coding region was present in tissue from both patients with ovarian cancer and from

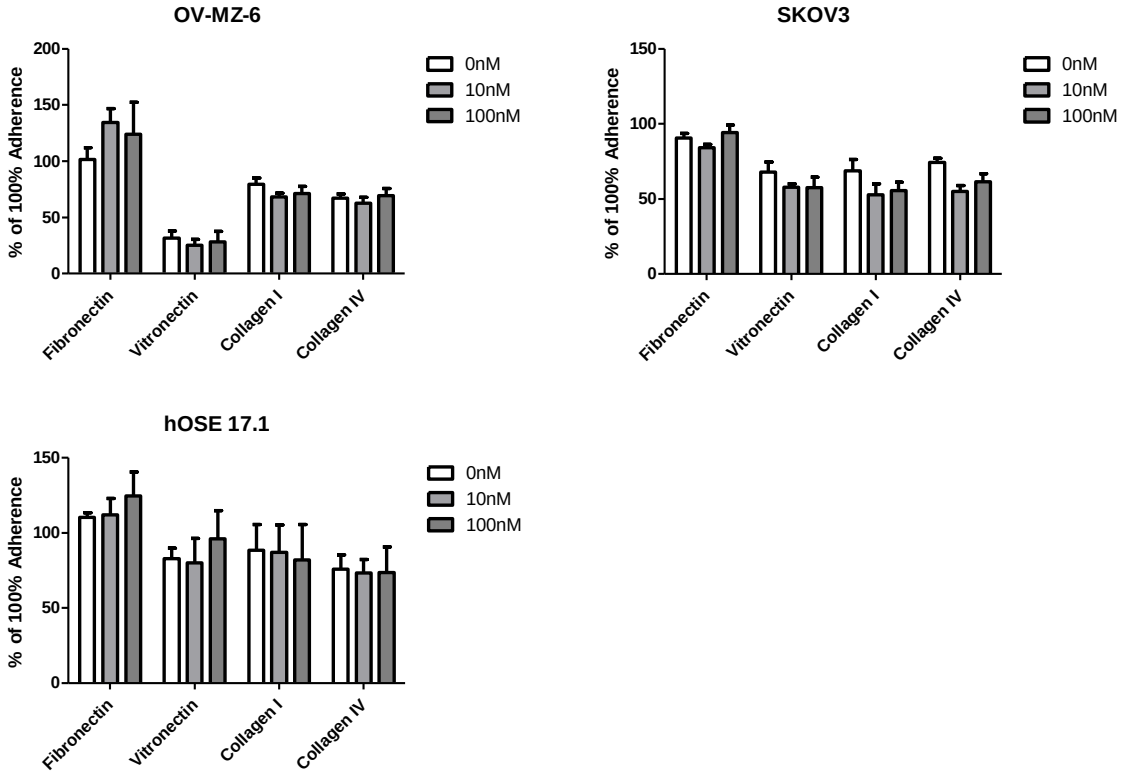

Figure 4.6 Attachment assays for SKOV3 and OV-MZ-6 ovarian cancer cell lines and the hOSE 17.1 normal ovarian cell line in response to obestatin treatment using a CyQuant assay. Results are shown as a mean percentage (minus background) of the total number of cells that could possibly adhere to the uncoated 100% adherence plate with the aid of serum +/- SEM. No statistically significant differences were seen. n = 3 repeats with 3 replicates per independent experiment.

samples of normal ovary. Obestatin mRNA levels, however, were not significantly different between the normal and cancer tissue. Similar findings have been reported for other small growth factor peptides, such as kisspeptin (or mestatin) [567], which suggest a favorable prognosis in ovarian clear cell cancer [568]. Epidermal growth factor (EGF) [569], which stimulates migration in ovarian cancer, and its receptor have potential as therapeutic targets for this disease [570, 571]. The normal tissue in the current study, however, was unlikely to contain ovarian surface epithelium, from which most ovarian cancers develop. In the previous chapter (3.3.1) it was shown that all cell lines expressed the full length preproghrelin transcript and thus, have the potential to produce obestatin.

There have been few studies describing obestatin expression in the ovary, or in ovarian cancer. In a small study of patients who underwent surgery for benign ovarian tumours (22 patients), or ovarian cancer (31 patients), and in 32 control women, obestatin was shown to be elevated in the plasma of patients with both benign ovarian neoplasms and ovarian cancers [114]. These authors hypothesised that obestatin could be a useful biomarker for ovarian cancer [114]. There were a number of limitations to this study, however. It is unclear whether these blood samples were taken before, after or during surgery. Postoperative histopathology of the removed tumours revealed that 7 of the benign cases and 15 of the ovarian cancer cases were variations of the serous phenotype.

Using immunohistochemistry, we demonstrated that normal ovarian surface epithelium expresses very low levels of obestatin, while immunoreactivity ranged from low to moderate levels in ovarian cancer samples. Obestatin peptide expression may, therefore, be elevated in ovarian cancer compared to normal ovarian surface epithelium. The antibody used in this study was raised against the obestatin peptide, which is also present in the proghrelin peptide and the C-ghrelin peptide prior to further processing. Obestatin immunoreactivity in the ovary is likely to be due to the presence of the mature form of obestatin, as it is unlikely that ghrelin processing intermediates would accumulate in the cells. Further studies using mass spectrometry or Western analysis are required in order to determine whether the mature form of obestatin is expressed by ovarian cancer cells. Obestatin expression has also been demonstrated, in a study of a range of serous ovarian cancers, benign ($n = 20$),

borderline (n = 7) and malignant (n = 20) serous tumours, using immunohistochemistry [380]. Further studies on larger sample sets and other types of ovarian cancers are required in order to determine if obestatin expression is elevated in ovarian cancer compared to normal ovarian surface epithelium.

In the current study expression of obestatin was also demonstrated in the normal human corpus luteum, indicating that it may play a normal physiological role. The SKOV3 and OV-MZ-6 serous ovarian cancer cell lines and hOSE 17.1 normal ovarian cells also express preproghrelin mRNA, which contains the coding region for obestatin. Obestatin was highly expressed in inflammatory cells in a case of salphingitis, indicating that it may play a role in the immune response in the reproductive tract and other tissues. It is possible that novel obestatin-specific transcripts are also produced and these could influence the ghrelin to obestatin ratio [118].

In this study, it has been demonstrated for the first time that obestatin may play a role in ovarian cancer migration and invasion through the extracellular matrix, and we hypothesise that obestatin may play a similar role in other cancers. In this study the migration was increased SKOV3 and OV-MZ-6 ovarian cancer cell lines, however, obestatin had no effect on cell migration in the hOSE 17.1 normal ovarian cell line. This suggests that the response may be cancer specific and occurs in cells that have a tendency to migrate; however, further investigation with a wider range of cell lines is required to confirm this hypothesis. Surprisingly, obestatin treatment increased invasion through Matrigel in the hOSE 17.1 normal ovarian cell line. Cell invasion was also increased in the OV-MZ-6 ovarian cancer cell line, but not in the SKOV3 cancer cell line. This may be suggests that obestatin may also have some roles in normal ovarian surface epithelium, in addition to a role in ovarian cancer progression. The very limited effects on invasion in the ovarian cancer cell lines, compared to the significiant increases observed in the normal hOSE 17.1 cell line, indicate that obestatin could be more involved in cell motily in ovarian caner metastasis rather than degradation of extracellular matrix to allow invasion into a new site. In ovarian cancer metastasis, metastatic cells are sloughed off as spheroids from the primary tumour into the peritoneal cavity and this does not require tissue invasion [528]. Migration and cell motility is therefore more important in the early

stages of cancer dissemination, than the ability to degrade and invade [551].

Since its discovery, obestatin has been shown to have a number of functions, some of which may be relevant in cancer [7]. Obestatin stimulates cell proliferation [10-12, 465] which is a hallmark of cancer development and progression [393]. A major characteristic of cancer cells is an ability to be self-sufficient in growth signals, while being insensitive to growth-inhibitory signals. In cancer, many oncogenes mimic normal growth signalling to stimulate proliferation [393]. Obestatin can stimulate cell proliferation in normal retinal epithelial cells [10] and the HIT-T15 and INS-1E pancreatic β-cells [12]. In cancer cells, obestatin also increased proliferation in the KATO-III gastric cancer cell line [11], but in contrast, proliferation was reduced with obestatin treatment in the TT thyroid carcinoma cell line [56]. In this study, in normal and ovarian cancer cells, obestatin was shown to have no significant effect on proliferation using the WST-1 and CyQuant assays. These results contrast with another recent study in porcine ovarian granulosa cells that suggested that obestatin stimulated cell proliferation [465] and this was determined by measuring expression of the PCNA (proliferating cell nuclear antigen) and cyclin B1 proliferation markers and MAP kinase expression [465]. Direct proliferation assays were not performed in this study. These cells are derived from the stroma surrounding the follicle, however, and have a very different origin to ovarian cancer cells which are derived from the ovarian surface epithelium. Obestatin appears to have different effects on proliferation in different cell types.

Cell attachment is important in ovarian cancer progression, as it allows the ovarian cancer cells that have shed into the peritoneal cavity to adhere to the mesothelium that lines the cavity, and this is its primary site of metastasis [527, 528]. The metastasised cells attach to the mesothelium by binding to ECM molecules (including fibronectin, vitronectin and collagen I) which are secreted on the surface of the mesothelial cells [552-554]. As the role of obestatin in promoting cancer cell attachment has not previously been investigated, the ability of obestatin to influence adhesion to the major ECM components, fibronectin, vitronectin, collagen I and collagen IV was measured. It was demonstrated that obestatin had no significant effect on adhesion in the normal or cancer ovarian cell lines. This finding is corroborated by a single study that investigated attachment of monocytes in the

development of atherosclerosis. Obestatin has previously been shown to decrease the expression of vascular cell adhesion molecule-1 in monocytes, but did not affect adhesion itself [555]. Obestatin appears to have no effect on attachment of ovarian cancer cells.

We have demonstrated that ghrelin (Chapter 3.3.1) and the obestatin-coding region are expressed at the mRNA level, and that the obestatin peptide is expressed at a protein level in ovarian cancer [45]. This is the first report showing that the ghrelin-gene derived peptide, obestatin, is expressed at an mRNA level in the normal ovary and in ovarian tumours, and that obestatin promotes cancer cell migration *in vitro*. We have demonstrated for the first time that obestatin can enhance invasion in the hOSE 17.1 normal human surface epithelial derived cell line and the OV-MZ-6 ovarian cancer cell line. These data, provide further evidence that obestatin is a hormone with functions outside the endocrine brain-gut axis.

CHAPTER 5

The effect of obestatin on protein expression in ovarian cancer cell lines

5.1 Introduction

There is increasing evidence that, like ghrelin, obestatin may act as an autocrine/paracrine factor in a number of systems, or it could act as an endocrine hormone on a range of peripheral tissues [572]. In the previous chapters it has been demonstrated that obestatin and ghrelin promote migration in ovarian cancer cell lines. As this increase in cell migration could be significant in ovarian cancer progression, it is important that a greater understanding of its mechanism of action is attained. Thus, in this chapter we endeavoured to further investigate the potential downstream targets, pathways and processes that may be stimulated by obestatin treatment in ovarian cell lines.

Currently obestatin is known to stimulate proliferation and it has anti-apoptotic effects through the ERK1/2 MAPK signalling pathway [10-12, 462, 465, 466]. Obestatin signals through activation of Akt to inhibit apoptosis [12] and regulate adipocyte metabolism and adipogenesis [464]. An investigation into obestatin-stimulated Akt signalling in the KATO-III and AGS gastric cancer cell lines revealed that obestatin induced an association between GPR39 and a β-arrestin 1/Src signalling complex, resulting in transactivation of the epidermal growth factor receptor (EGFR), which then activated Akt [472]. This was paralleled by the phosphorylation of mTOR (mammalian target of rapamycin), which can lead to Akt phosphorylation, or can occur downstream of Akt phosphorylation [472]. Although this study indicated that GPR39 was involved in obestatin signalling, a number of studies have shown that obestatin does not bind GPR39, demonstrating that GPR39 is not the obestatin receptor [452-455]. In studies of human retinal epithelial cells and the KATO-III gastric cancer cell line, obestatin enhanced proliferation and stimulated ERK1/2 signalling, through the activation of Gi, PI3K, protein kinase C (PKC) and Src [10, 11]. Obestatin-stimulated growth hormone secretion in rat tumour somatotroph cells is also mediated by these signalling pathways [466]. The cardioprotective effects of obestatin in rat H9c2 cardiac cells and isolated ventricular myocytes are mediated by PI3K, PKC and ERK1/2 [462]. Obestatin has also been demonstrated to signal through adenylyl cyclase/cAMP/protein kinase A (PKA) [12] and AMPK [464] pathways.

In order to investigate the processes and pathways stimulated by obestatin in ovarian

cancer, we used two dimensional-difference in gel electrophoresis (2D-DIGE). 2D-DIGE is a broad spectrum, discovery-driven proteomics approach for comparing and analysing complex protein mixtures in multiple samples. 2D-DIGE is a fluorescent gel based method where samples, labelled by dyes (such as Cy3 or Cy5 dye), are initially separated by native charge in the first dimension using isoelectric focusing (IEF), and then separated by molecular mass in the second dimension using SDS-PAGE [573]. Unlike other 2D gel methods, an internal Cy2 dye labelled standard control sample can also be used. For this method, the internal standard is a combination of all the samples being analysed, to allow for both accurate analysis of in gel differential expression and comparison of targets across gels [574].

In this study, we identified 26 proteins which were differentially expressed in response to obestatin treatment in the SKOV3 ovarian cancer cell line, and 15 of these were regulated by obestatin in the hOSE 17.1 normal ovarian cell line. Further analysis of differentially expressed proteins in response to obestatin treatment was performed using Ingenuity Pathway Analysis software and revealed that the ERK1/2 pathway may play a central role in obestatin function.

5.2 Methods

5.2.1 Cell lines and cell culture

In this study, assays were performed using the SKOV3 and the OV-MZ-6 human epithelial ovarian serous adenocarcinoma cell lines [510, 511] and the hOSE 17.1 normal surface epithelium-derived cell line [512]. The SKOV3, OV-MZ-6 and hOSE 17.1 cell lines used in this study were propagated as described in the General Methods, Chapter 2.1.

5.2.2 Two Dimensional-Difference in Gel Electrophoresis (2D-DIGE)

A 12 gel 2D-DIGE (GE Healthcare, Buckinghamshire, UK) experiment was used to investigate the differential expression of proteins in response to obestatin treatment (10 or 100 nM obestatin), or a no treatment control, in the hOSE 17.1 normal ovary derived cell line and the SKOV3 ovarian cancer cell line. The SKOV3 and hOSE 17.1 cell lines were passaged into T175 flasks (Nunc) and allowed to attach for 24 h. Cell lines were treated for 3 days with either medium only, or obestatin (10 nM and

100 nM; Auspep) and obestatin was replenished every 24 hours. After 3 days of treatment, cells were removed from the flask surface by cell scraping and collected into 800 µL 2D-DIGE extraction buffer (30 mM Tris, 7 M urea, 2 M thiourea, 4% CHAPS pH 8.8). Cell lysates were collected on ice and vortexed several times prior to storage at -80 ºC. Four independent replicates were prepared. Cell lysates were precipitated using the Ettan 2-D Clean Up Kit (GE Healthcare), and resuspended according to the manufacturer's instructions. The protein concentrations of cell lysates for DIGE analysis were determined using a Bradford assay (BioRad) according to the manufacturer's instructions. 2D-DIGE experiments were performed as previously described [575] and according to the manufacturer's instructions. An internal standard which contained pooled, equal aliquots of all samples in the experiment (with aa total protein content of 600 µg) was prepared and labelled with Cy2 dye (GE). Protein test samples (50 µg) were labelled with 400 pmoles either Cy3 or Cy5 dye (GE Healthcare) for 1 h on ice in the dark, according to the manufacturer's instructions. This reaction was stopped by quenching with 1 µL 10 mM lysine (and 12 µL for the internal standard). A total of 24 test samples were labelled, with four replicates of each treatment of each cell line. Cross labelling of replicate samples (two replicates with Cy3 and two replicates with Cy5) was performed to avoid any bias in subsequent analysis due to labelling. For each gel, one Cy3 and one Cy5 labelled sample, with an equal amount of Cy2-labelled internal standard, were combined in rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mM DTT, 0.5% immobilised pH gradient (IPG) buffer pH 3-11 non-linear) to a final volume of 450 µL.

5.2.3 Two-Dimensional Electrophoresis, gel imaging and DIGE analysis

In the DIGE experiment, and for preparative gels for the MS analysis of proteins of interest, Immobiline Dry Strip immobilised pH gradient gels (IPG) (GE Healthcare), pH 3-11 non-linear (NL) gradient strips (24 cm) were used. Strips were rehydrated with 450 µL each sample, covered with Plus One Dry Strip Cover Fluid (GE Healthcare) and incubated at RT for 20 hours in the dark. Following rehydration, the IPG gels were focussed on an Ettan IPGphor 3 isoelectric focusing unit (GE Healthcare) at 500 V for 1 h with a gradient to 1000 V over 7 h, followed by a gradient to 10 000 V over 3 h, and then 10 000 V for 4 h. On completion of the first-dimension IEF, IPG gels were reduced and alkylated (with 1% DTT in rehydration

buffer for 15 min, followed by 2% iodoacetamide in rehydration buffer for 15 min). Second dimension protein separation was performed using 12% SDS-PAGE gels (375 mM Tris HCl, pH 8.8, 0.1% SDS) using an Ettan DALTsix electrophoresis system (GE Healthcare). The strips were embedded on the top of the gels with 0.5% agarose (in Laemmli buffer, 25 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS, pH 8.3) and electrophoresed at room temperature (at 2 W/gel for 1 h followed by 4-5 h at 12 W/gel).

Gel images of the combined Cy2, Cy3 and Cy 5 labelled gels were scanned using a Typhoon TRIO Variable Mode Imager (GE Healthcare) using excitation/emission wavelengths of 488/520 nm for Cy2, Cy3 532:580 nm and Cy5 633:670 nm. Images were initially cropped using ImageQuant TL software (GE Healthcare) prior to final image analysis to remove any non-essential information from the image files (including gel edges and background image of the scanner). Final image analysis was performed using DeCyder 2D software (GE Healthcare) (as per the manufacturer's instructions) using both Differential-In-gel Analysis (DIA) and Biological Variation Analysis (BVA) modules (Fig 5.1). The DeCyder 2D software is specially designed for the Ettan DIGE system and is an automated image analysis software suite that allows for detection, quantitation, positional matching and differential protein abundance analysis. The DIA analysis detected protein spots on the set of images for each gel image and quantified them. Ten proteins were set as landmarks to allow the correct alignment of Cy3 and Cy5 scans of each gel to their internal standard Cy2 scan. This part of the software also subtracts background, performs in-gel normalisation and removes artefacts. After processing the image sets for each gel in the DIA, the BVA module was used to compare differential protein abundance across the gels. From the BVA analysis, 42 spot were selected for picking from the preparative gels. Spots were chosen on the basis of having large positive or negative average ratios (differential expression) when comparing the treated samples to their media control.

5.2.4 Preparative gels, protein spot picking and preparation of spots for mass spectrometry

In order to maximise the success rate of protein sequencing spots of interest, (that have been identified using DIGE), are extracted from a preparative gel where an

excess amount of protein has been loaded. Six samples (including 2 samples of SKOV3 and hOSE 17.1 cells treated with 100 nM obestatin treatment, and one sample from each cell line treated with medium only), that represented all the spots of interest overall, were electrophoresed using the preparative gel, with 1 mg of each sample loaded on separate gels. Preparative gels were prepared as described above (5.3.2), and stained with Colloidal Coomassie Stain (0.08% Coomassie Brilliant Blue G-250 (CBB G250), 1.6% orthophosphoric acid, 8% ammonium sulphate and 20% methanol) [576]. Protein spots of interest were excised from the gels, washed twice in MilliQ water, then washed twice with a solution containing 25 mM ammonium bicarbonate and 50% Acetonitrile (ACN) (HPLC grade) followed by drying under vacuum at 30ºC. The desiccated gel pieces were rehydrated in 15 mL digestion buffer (25 mM ammonium bicarbonate, 1 mM calcium chloride) containing 20 ng/mL sequencing grade modified trypsin (Promega, Madison, WI) for in-gel digestion [576]. Peptide extracts were dried using evaporative centrifugation and reconstituted in 25 µL 5% formic acid. The reconstituted extracts were sonicated for 10 min prior to LC-MS/MS analysis.

5.2.5 Mass spectrometry

5.2.5.1 Statement of contribution for mass spectrometry

The mass spectrometry analysis of the 2D-DIGE picked spot samples described in this section was performed by Peter Josh (CSIRO, Qld).

5.2.5.2 LC-MS/MS analysis and database searching

Peptide extracts were injected into a Shimadzu Prominence Ultra Fast Liquid Chromatograph (UFLC) system (Shimadzu, Kyoto, Japan) at a flow rate of 3 µL/min with a Vydac MS C18 300 Å column (150 mm x 300 µm), with a particle size of 5 µm (Grace Davison, Deerfield, USA). Chromatographic separation was developed using a linear gradient of 2-45% solvent B (90% acetonitrile/10% water containing 0.1% formic acid) in solvent A (0.1% formic acid) over 40 minutes. The HPLC eluent was coupled directly to a QStar Elite Hybrid liquid chromatography system coupled with tandem mass spectrometry (LC-MS/MS) (Applied Biosystems/MDS Sciex, Foster City, USA) fitted with a nano-electrospray ionization source. Source conditions included an ion spray voltage of 3400 V, nebuliser gas flow 20, curtain

gas flow 15 and interface heater temperature 120°C. Collision-induced dissociation settings included collision-activated dissociation (CAD) gas 4 and a declustering potential of 70 V. Data was collected in information-dependent acquisition mode. Following an MS survey scan (*m/z* 350-1800), MS/MS measurements were performed for the four most intense precursor ions (50 counts/s threshold, +2 to +5 charge state and *m/z* 100-1600). Data was acquired and processed using Analyst QS 2.0 software.

ProteinPilot 3.0 software (Applied Biosystems) with the Paragon Algorithm, was used for the identification of proteins. Data was searched against the NCBI non-redundant database. Search parameters included cysteine alkylation with iodoacetamide and trypsin as the digestion enzyme, and there was no restriction on taxonomy. Single amino acid substitutions and biological modifications were allowed. The software allows 126 possible biological modifications including acetylation, phosphorylation and methylation. Protein scores (a measure of the increase in evidence specific to each protein raising confidence in its correct identification) >1.3 with a confidence interval (CI) >95% were considered to be indicative of a positive identification.

5.2.6 Pathway analysis

5.2.6.1 Statement of contribution for Ingenuity Pathway Analysis

The pathway analysis using the Ingenuity Pathway Analysis software described in this section was performed by Dr Inge Seim and Peter Josh.

5.2.6.2 Ingenuity Pathway Analysis

The Ingenuity Pathway Analysis tool (IPA, Ingenuity Systems, Redwood, CA) was used to examine the differentially expressed proteins identified from the SKOV3 cell line using ProteinPilot. The IPA tool analyses the input protein list in the context of known molecular and chemical interactions, cellular phenotypes, and disease processes. The list of proteins which were differentially expressed in response to obestatin treatment (compared to untreated cells) were mapped to the Ingenuity database and ranked by score. The score indicates the likelihood that the proteins grouped together in the network were discovered together by chance, with a 99%

confidence level, and scores ≥3 are considered to be significant.

5.2.7 Investigation of ERK1/2 as a potential pathway for migration in SKOV3 and OV-MZ-6 ovarian cancer cell lines

5.2.7.1 Western immunoblotting using anti-active antibodies

As pathway analysis studies demonstrated that the ERK1/2 protein expression may be a central pathway mediating the effects of obestatin treatment, Western analysis was performed in the ovarian cancer cell lines to determine if obestatin treatment also activates this signalling pathway, leading to ERK1/2 phosphorylation. We investigated whether or not ERK1/2 phosphorylation was associated with increased cell migration in response to obestatin (as observed in Chapter 4). Western analysis was performed as previously described (Chapter 2.7) using an anti-active antibody for phosphorylated ERK1/2 (Cell Signalling, Genesearch, Arundel, Qld, Australia) to determine if obestatin treatment activates this signalling pathway. SKOV3 and OV-MZ-6 ovarian cancer cell lines were treated with obestatin (0, 0.1, 1, 10,100 and 1000 nM) and ERK1/2 signalling was measured at 15 min for SKOV3 and 30 min for OV-MZ-6. These timepoints were predetermined as they gave peak ERK1/2 signalling in these cell lines (described in Chapter 2).

5.2.7.2 Investigation of ERK1/2 using Sigma Phospho-Erk1 ($pThr^{202}/pTyr^{204}$) + Erk2 ($pTyr^{185/187}$) and pan-Erk1/2 ELISA Kit

To further investigate the effects of obestatin treatment on ovarian cancer cell lines using a more quantitative method, an anti-active ERK1/2 ELISA was performedin the SKOV3 ovarian cancer cell line using the Sigma phospho-ERK1/2 ELISA Kit (Sigma-Aldrich). SKOV3 ovarian cancer cells were seeded at 3 $x10^5$ cells/well in 6-well plates. After 8h, cells were incubated in phenol red free medium without FCS overnight. To investigate ERK1/2 signalling, cells were treated with obestatin (0, 10 or 100 nM) and incubated for 0, 5, 10, 15 and 30 min. At each time-point media was aspirated, 250 µl lysis buffer (kit lysis buffer plus 50 nM NaF, complete protease inhibitor cocktail, Roche and Phosphatase Inhibitor Cocktail 2, Sigma-Aldrich) was added per well and the plates were placed on ice. At the end of the time course, plates were then incubated for 30 min at 4°C shaking, the lysates were transferred to tubes, centrifuged (10000 *x* g for 10 min at 4 ºC) and supernatant transferred to new

tubes. Samples were diluted 5 fold and duplicate wells per sample for phosphorylated and pan ERK1/2 were assayed. The ELISA assay was performed as per manufacturer's instructions and absorbance was determined (450 nm) using a Benchmark Plus Microplate Spectrophotometer System (BioRad).

5.2.8 Statistics

Statistical analyses were undertaken as described in Chapter 2.8.

5.3 Results

5.3.1 Differential protein expression in response to obestatin treatment compared to control in the SKOV3 ovarian cancer cell line

A proteomic, 2D-DIGE LC-MS/MS method was applied to the SKOV3 ovarian cancer cell line and the hOSE 17.1 normal ovary-derived cell line in order to identify proteins that are differentially regulated by obestatin treatment (Fig. 5.1). We identified 26 proteins that were differentially regulated by obestatin treatment compared to no treatment controls in the SKOV3 ovarian cancer cell line (Table 5.1). In the hOSE 17.1 cell line, 15 of the 26 proteins were found to be differentially expressed using the DeCyder software. A number of these 26 proteins have been previously associated with cell migration, which was shown to be stimulated by obestatin treatment in this thesis (Chapter 4, Tables 5.2 and 5.3). Many of the other proteins identified were associated with processes that could affect cell migration, such as remodelling of the cytoskeleton and regulation of protein expression.

Ingenuity Pathway Analysis (IPA) was performed in order to place the differentially regulated proteins in a biological network and to determine the relationship between these proteins (Fig 5.2). Two networks were generated by IPA analysis, using a 99% confidence level (score ≥ 3) that peptides in a particular network were not networked together due to chance. The proteins identified, their major pathway functions and their associated networks are shown in Table 5.4. Pathway analysis demonstrated that the differentially regulated proteins were associated with cancer progression and post-translational modifications. The MAPK signalling protein, ERK1/2, was identified as an important protein that interconnected 16 of the 26 proteins identified through the 2D-DIGE LC-MS/MS analysis based on the software's analysis of

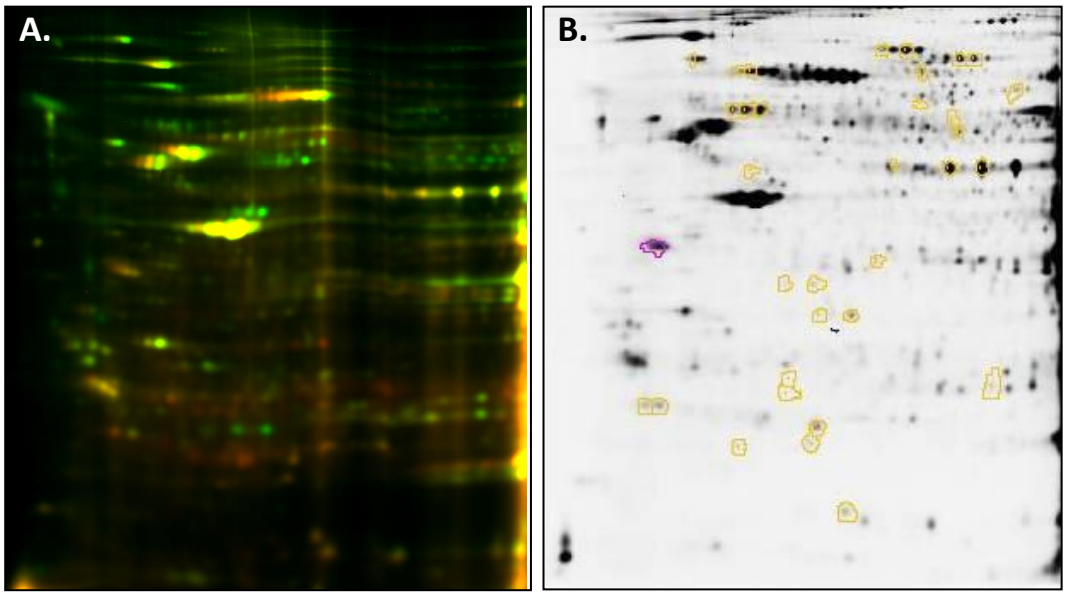

Figure 5.1 Representative 2D-DIGE gels showing a combined image for analysis showing Cy3 and Cy5 fluorescencently-labelled proteins (from 2 different samples) and (B) a representative DeCyder software-generated image showing protein spots that have been selected for further analysis after removal from the gel (circled proteins in B). A. Representative 2D-DIGE gel of protein from the SKOV3 cancer cell line (labelled with Cy) and the hOSE 17.1 normal ovarian cell line (labelled with Cy dye) and treated with medium alone. Red and green protein spots represent differentially expressed proteins, while yellow spots represent proteins with the same abundance in the two samples that are being compared. B. Representative gel image of 2D gel (of SKOV3 cells treated with medium only) was created and analysed using DeCyder image software (GE). DeCyder software identified proteins differentially expressed in response to 72 h obestatin treatment (circled), compared to medium only treated controls. These proteins were excised from preparative gels and identified using liquid chromatography tandem mass spectrometry (LC-MS/MS).

Table 5.1 Proteins that were differentially regulated in the SKOV3 ovarian cancer and hOSE 17.1 normal ovarian cell lines in response to obestatin treatment compared to untreated controls. The effects of obestatin treatment on protein expression in the cell lines compared to their respective untreated controls are expressed as a positive or negative average ratio (representing the degree of difference in the standardized abundance between two groups being compared). SKOV3 and hOSE 17.1 were treated with 0, 10 and 100 nM obestatin treatments over 72 h. ↓ = proteins downregulated by obestatin treatment compared to untreated controls; ↑ = proteins upregulated by obestatin treatment compared to untreated controls. ↔ = proteins that were unchanged by the obestatin treatment.

	Comparison to 0 nM (Media Only) Treatment			
Protein ID	hOSE 17.1 10 nM	hOSE 17.1 100 nM	SKOV3 10 nM	SKOV3 100 nM
60 kDa heat shock protein, mitochondrial	↓ (-2.44)	↑ (1.14)	↓ (-3.65)	↔ (1.10)
78 kDa glucose-regulated protein	↔ (1.60)	↑ (2.35)	↓ (-5.24)	↓ (-1.34)
Actin-related protein 2/3 complex subunit 5-like protein	↔ (1.12)	↔ (1.16)	↑ (1.56)	↑ (2.28)
Alpha-enolase	↓ (-1.32)	↔ (1.15)	↓ (-2.39)	↔ (1.06)
Annexin A2	↓ (-1.53)	↔ (1.11)	↓ (-1.34)	↑ (1.20)
Annexin A4	↔ (-1.00)	↓ (-1.14)	↑ (1.23)	↑ (1.86)
Carboxypeptidase D	↓ (-14.13)	↔ (-1.11)	↓ (-6.60)	↓ (-2.68)
Cofilin-1	↔ (1.12)	↔ (1.16)	↑ (1.56)	↑ (2.28)
Elongation factor 1-delta	↔ (-1.01)	↔ (-1.01)	↓ (-1.17)	↔ (1.02)
Eukaryotic initiation factor 4A-I	↔ (-1.09)	↔ (-1.01)	↓ (-1.58)	↔ (1.06)
Glutathione S-transferase P	↔ (-1.03)	↔ (1.08)	↑ (1.47)	↔ (1.45)
Heat shock cognate 71 kDa protein	↓ (-1.52)	↔ (-1.08)	↓ (-2.87)	↔ (-1.06)
Heat shock protein HSP 90-alpha	↔ (-2.65)	↔ (-1.06)	↓ (-9.79)	↓ (-1.66)
Heat shock protein HSP 90-beta	↔ (-2.65)	↔ (-1.06)	↓ (-9.79)	↓ (-1.66)
Heterogeneous nuclear ribonucleoprotein K	↓ (-2.44)	↑ (1.14)	↓ (-3.65)	↔ (1.10)
Heterogeneous nuclear ribonucleoproteins C1/C2	↔ (-1.01)	↔ (-1.01)	↓ (-1.17)	↔ (1.02)
Inorganic pyrophosphatase	↓ (-1.32)	↑ (1.11)	↓ (-1.29)	↔ (1.07)
Nucleophosmin	↔ (-1.01)	↔ (-1.01)	↓ (-1.17)	↔ (1.02)
Peroxiredoxin-2	↑ (2.00)	↔ (-1.00)	↑ (2.14)	↓ (-1.45)
Proteasome subunit alpha type-5	↑ (1.23)	↔ (1.18)	↑ (2.17)	↑ (1.97)
Pyruvate kinase isozymes M1/M2	↓ (-2.44)	↑ (1.14)	↓ (-3.65)	↔ (1.10)
Thioredoxin-dependent peroxide reductase, mitochondrial	↔ (-1.03)	↔ (1.08)	↑ (1.47)	↔ (1.45)
Tubulin alpha-1C chain	↓ (-5.87)	↓ (-1.62)	↓ (-7.20)	↓ (-2.01)
Tubulin beta chain	↔ (-1.00)	↓ (-1.14)	↑ (1.23)	↑ (1.86)
Ubiquitin carboxyl-terminal hydrolase isozyme L1	↔ (-1.08)	↔ (-1.06)	↑ (4.19)	↑ (2.28)
Vimentin	↓ (-5.87)	↓ (-1.62)	↓ (-7.20)	↓ (-2.01)

Table 5.2 Proteins that were downregulated in response to obestatin treatment compared to untreated controls in the SKOV3 ovarian cancer cell line. Proteins that were downregulated are shown as a negative average ratio (the degree of difference in the standardized abundance between two groups or populations being compared). The SKOV3 cell line was treated with 10 nM obestatin for 72 h. The major functions of the proteins and their role in cell migration are listed. ↓ = proteins downregulated by obestatin treatment compared to untreated controls. Information available regarding the known protein functions is summarised from Gene, Pubmed (http://www.ncbi.nlm.nih.gov/gene). The relationship between the identified protein and cell migration is summarised from the literature. N= no relationship with migration has been identified in the literature.

Protein (gene)	Avg. ratio	Protein function (taken from Gene Pubmed)	Relationship to Migration	Reference
Alpha-enolase (ENO1)	↓ (-2.39)	NM_001428.3/NP_001419.1 One of three enolase isoenzymes found in mammals which function as glycolytic enzymes. In tumour cells, it is upregulated, cell surface expressed and supports anaerobic proliferation and promotes cancer invasion. A shorter isoform functions as a tumour suppressor. Gene ID: 2023, updated on 9-Sep-2012	Inhibition of alpha-enolase inhibits cell migration and invasion in the MDA-MB-231 breast cancer cell line by inhibiting secretion of a subset of cytokines, and suppressing plasminogen activation	[577, 578]
Annexin A2 (ANXA2)	↓ (-1.34)	NM_001002858.2/ NP_001002858.1 Belongs to the annexin family of calcium-dependent phospholipid binding proteins. Members of this family play a role in the regulation of cellular growth and in signal transduction pathways. This protein functions as an autocrine factor which heightens osteoclast formation and bone resorption. Gene ID: 302, updated on 15-Sep-2012	Annexin A2 knockdown decreased glioma cell line and MDA-MB-231 cell line migration. In another study annexin A2 induced increased migration of glioma cells toward neural stem cells and glioblastoma cells in Boyden chamber migration assays.	[579-581]
Carboxypeptidase D (CPD)	↓ (-6.60)	NM_001304.4/NP_001295.2 Part of the metallocarboxypeptidase family of enzymes with a subfamily classification of a regulatory B-type carboxypeptidase.	Expressed in basophil invasion	[582, 583]

Protein (gene)	Avg. ratio	Protein function (taken from Gene Pubmed)	Relationship to Migration	Reference
		Functions in the processing of proteins that transit the secretory pathway Gene ID: 1362, updated on 15-Sep-2012		
Elongation factor 1-delta (EEF1D)	↓ (-1.17)	NM_001130053.2/ NP_001123525.2 A subunit of the elongation factor-1 complex, which is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome. Functions as guanine nucleotide exchange factor. Gene ID: 1936, updated on 9-Sep-2012	N	
Eukaryotic initiation factor 4A1 (EIF4A1)	↓ (-1.58)	NM_001416.3/NP_001407.1 ATP-dependent RNA helicase which is a subunit of the eIF4F complex involved in cap recognition and is required for mRNA binding to ribosome. Gene ID: 1973, updated on 15-Sep-2012	Differentially expressed in human colorectal carcinoma cell lines with different metastatic potentials.	[584]
78 kDa Glucose-regulated protein (GRP78/ HSPA5)	↓ (-5.24)	NM_005347.4/NP_005338.1 A member of the heat shock protein 70 (HSP70) family. It is localized in the lumen of the endoplasmic reticulum (ER), and is involved in the folding and assembly of proteins in the ER. It is a potent anti-apoptotic protein and plays a critical role in tumour cell survival, tumour progression and angiogenesis, metastasis and resistance to therapy. Gene ID: 3309, updated on 23-Sep-2012	Silencing of GRP78 in head and neck cancer cell lines inhibits cell migration. Downregulation of GRP78 in hepatocellular carcinoma enhances cell migration.	[585-589]
60 kDa Heat shock protein, mitochondrial (HSP60/ HSPD1)	↓ (-3.65)	NM_002156.4/NP_002147.2 HSP60 is a mitochondrial protein and member of the chaperonin family. It is essential for the folding and assembly of newly imported proteins in the mitochondria It has been associated with carcinogenesis, specifically with tumour cell survival and proliferation.	Knockdown of HSP60 in head and neck cancer cell lines enhances cell migration. Consistently, treatment of T cells with HSP60 inhibited downstream effects of processes required for cell migration. Contrastingly, treatment with bacterial HSP60 in gastric cancer cells and epithelial cells also increases migration.	[588, 590-593]

Protein (gene)	Avg. ratio	Protein function (taken from Gene Pubmed)	Relationship to Migration	Reference
		Gene ID: 3329, updated on 16-Sep-2012		
Heat shock cognate 71 kDa protein/ Heat shock 70kDa protein 8 (HSPA8)	↓ (-2.87)	NM_006597.4/NP_006588.1 A constitutively expressed member of the heat shock protein 70 family. Functions as a chaperone, and binds to nascent polypeptides to facilitate correct folding. Also functions as an ATPase in the disassembly of clathrin-coated vesicles during transport of membrane components through the cell. Gene ID: 3312, updated on 18-Sep-2012	N	
Heat shock protein HSP 90-alpha (HSP90AA1)	↓ (-9.79)	NM_001017963.2/NP_001017963.2 An inducible molecular chaperone that functions as a homodimer. It aids in the proper folding of specific target proteins by use of an ATPase activity that is modulated by co-chaperones. Gene ID: 3320, updated on 9-Sep-2012	HSP90 is essential for CB2-mediated signaling of cell migration in human embryonic kidney 293 cells. Mediates MMP-2 activation and increases breast cancer cell migration and invasion.	[594, 595]
Heat shock protein HSP 90-beta (HSP90AB1)	↓ (-9.79)	NM_007355.2/NP_031381.2 A constitutively expressed member of the HSP90 proteins that are normally associated with other co-chaperones and play important roles in folding newly synthesized proteins or stabilizing and refolding denatured proteins after stress. Gene ID: 3326, updated on 25-Sep-2012	HSP90 is essential for CB2-mediated signaling for cell migration in human embryonic kidney 293 cells.	[594]
Heterogeneous nuclear ribonucleoprotein K (HNRNPK)	↓ (-3.65)	NM_002140.3/NP_002131.2 Belongs to the subfamily of ubiquitously expressed heterogeneous nuclear ribonucleoproteins (hnRNPs) that are associated with pre-mRNAs in the nucleus and appear to influence pre-mRNA processing and other aspects of mRNA metabolism and transport. This protein is also thought to have a role during cell cycle progression. Gene ID: 3190, updated on 25-Sep-2012	hnRNPK appears to be a multifunctional signaling protein in metastasis of cancer. Co-expression of HNRNPK with N-Wiskott-Aldrich syndrome protein (N-WASP) reverses the stimulation of cell spreading by N-WASP during cell adhesion and migration.	[596, 597]
Heterogeneous nuclear	↓ (-1.17)	NM_001077442.1/ NP_001070910.1	N	

Protein (gene)	Avg. ratio	Protein function (taken from Gene Pubmed)	Relationship to Migration	Reference
ribonucleo-proteins C1/C2 (HNRNPC)		Belongs to the subfamily of ubiquitously expressed heterogeneous nuclear ribonucleoproteins (hnRNPs) as hnRNP K above. This protein is involved in the assembly of 40S hnRNP particles. Gene ID: 3183, updated on 15-Sep-2012		
Inorganic pyrophosphatase (PPA1)	↓ (-1.29)	NM_021129.3/NP_066952.1 A member of the inorganic pyrophosphatase (PPase) family. PPases catalyze the hydrolysis of pyrophosphate to inorganic phosphate, which is important for the phosphate metabolism of cells. Gene ID: 5464, updated on 15-Sep-2012	N	
Nucleophosmin (NPM1)	↓ (-1.17)	NM_002520.6/NP_002511.1 This is a phosphoprotein which moves between the nucleus and the cytoplasm and is thought to be involved in several processes including regulation of the ARF/p53 pathway. Mutations in this gene are associated with acute myeloid leukemia. Gene ID: 4869, updated on 15-Sep-2012	Cleavage of nucleophosmin contributes to the limited motility, migration, and phagocytosis capabilities of resting macrophages. Nucleophosmin gene activating mutations promote NIH3T3 acute myeloid leukemia cell migration and invasion. Accumulation of NPM1 increased cellular migration, invasiveness, and colony formation in lung adenocarcinoma cell lines. Expression of MRJ (Mammalian relative of DnaJ) in breast cancer cell lines decreased migration and invasion and was correlated with reduced NPM1.	[598-601]
Pyruvate kinase isozymes M1/M2 (PKM2)	↓ (-3.65)	NM_002654.4/NP_002645.3 Large allosteric enzyme that regulates glycolysis. Interacts with thyroid hormones and may mediate their cellular metabolic effects. Gene ID: 5315, updated on 16-Sep-2012	N	
Tubulin alpha-1C chain (TUBA1C)	↓ (-7.20)	NM_032704.3/NP_116093.1 Part of the tubulin superfamily, alpha-tubulin, with beta-tubulin, make up the major components	N	

Protein (gene)	Avg. ratio	Protein function (taken from Gene Pubmed)	Relationship to Migration	Reference
		of microtubules Gene ID: 84790, updated on 15-Sep-2012		
Vimentin (VIM)	↓ (-7.20)	NM_003380.3/NP_003371.2 A member of the intermediate filament family. It is responsible for maintaining cell shape, integrity of the cytoplasm, and stabilizing cytoskeletal interactions. Also involved in the immune response, and controls the transport of low-density lipoprotein (LDL)-derived cholesterol from a lysosome to the site of esterification. It functions as an organizer of a number of critical proteins involved in attachment, migration, and cell signalling. Gene ID: 7431, updated on 23-Sep-2012	Marker of epithelial-mesenchymal transition (EMT). Up regulation of vimentin in hepatocellular carcinoma enhances cell migration. Correlates with EGFR and Stat3 activity to promote migration in ovarian cancer cells	[586, 587, 602-605]

Table 5.3 Proteins that were upregulated in the SKOV3 ovarian cancer cell line in response to obestatin treatment compared to untreated controls. The average ratio (the degree of difference in the standardized abundance between two groups or populations being compared) is shown. The SKOV3 cell line was treated with 10 nM obestatin for 72 h. The major functions of the proteins and their role in cell migration are listed. ↑= proteins that were upregulated by obestatin treatment compared to untreated controls. Information available regarding the known protein functions is summarised from Gene, Pubmed (http://www.ncbi.nlm.nih.gov/gene). The relationship between the identified protein and cell migration is summarised from the literature. N= no relationship to migration has been identified in the literature.

Protein (Gene)	Avg. ratio	Protein function (taken from Gene Pubmed)	Relationship to Migration	Reference
Actin-related protein 2/3 complex subunit 5-like protein (ARPC5L)	↑ (1.56)	NM_030978.1/NP_112240.1 Function not defined. May function as component of the Arp2/3 complex which is involved in regulation of actin polymerization and together with an activating nucleation-promoting factor (NPF) mediates the formation of branched actin networks Gene ID: 81873, updated on 9-Sep-2012	N	
Annexin A4 (ANXA4)	↑ (1.23)	NM_001153.3/NP_001144.1 Belongs to the annexin family of calcium-dependent phospholipid binding proteins. Functions are still not clearly defined, may be involved in exocytotic and endocytotic pathways. ANX4 has possible interactions with ATP, and has *in vitro* anticoagulant activity and also inhibits phospholipase A2 activity. Gene ID: 307, updated on 9-Sep-2012	Transient transfection of microRNA-7 into glioma cell lines causes inhibition of cell migration and invasion and suppression of tumorigenesis, 2-DE analysis identified down-regulated ANXA4 among the protein spots differentially expressed. In renal cell carcinoma over-expressed annexin IV promotes cell migration	[606, 607]
Cofilin-1 (CFL1)	↑ (1.56)	NM_005507.2/ NP_005498.1 A widely distributed intracellular actin-modulating protein that binds and depolymerizes filamentous F-actin and inhibits the polymerization of monomeric G-actin in a pH-dependent manner.	Cofilin-1 is involved in filopodium disassembly a process of cell migration. Increase in the level of cofilin in human colon adenocarcinoma cells, along with a reduction of inactive phosphorylated form of cofilin, was linked to increased migratory	[608-611]

Protein (Gene)	Avg. ratio	Protein function (taken from Gene Pubmed)	Relationship to Migration	Reference
		Gene ID: 1072, updated on 9-Sep-2012	activity. Decreased phospho-cofilin expression induced cell migration in human nasopharyngeal carcinoma cells. Phosphorylation and inactivation of CFL1 in basal breast cancer cell lines reduced cell motility.	
Glutathione S-transferase P (GSTP1)	↑ (1.47)	NM_000852.3/NP_000843.1 Glutathione S-transferases (GSTs) are a family of enzymes that play an important role in detoxification by catalyzing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione. GSTP1 variant proteins are thought to function in xenobiotic metabolism and play a role in susceptibility to cancer and other diseases. Gene ID: 2950, updated on 23-Sep-2012	Enhances migration in human breast cancer cell lines.	[612]
Peroxiredoxin-2 (PRDX2)	↑ (2.14)	NM_005809.4/NP_005800.3 A member of the peroxiredoxin family of antioxidant enzymes, which reduce hydrogen peroxide and alkyl hydroperoxides. This protein may play an antioxidant protective role in cells, and may contribute to the antiviral activity of CD8 (+) T-cells. This protein may have a proliferative effect and play a role in cancer development or progression. Gene ID: 7001, updated on 15-Sep-2012	Aloe-emodin (the main bioactive anthraquinones of Rheum palmatum) causes decreased cell migration along with enhanced oxidation of PRDX2 in HepG2 cells. PRDX2 is a negative regulator of PDGF signalling. Deficiency results in increased production of H_2O_2, enhanced activation of PDGF receptor and phospholipase Cgamma1, and increased cell migration.	[613, 614]
Proteasome subunit alpha type-5 (PSMA5)	↑ (2.17)	NM_002790.3/NP_002781.2 A proteasome is a multicatalytic proteinase complex that cleaves peptides in an ATP/ubiquitin-dependent process in a non-lysosomal pathway. This protein is a member of the peptidase T1A family. Gene ID: 5686, updated on 9-Sep-2012	N	
Thioredoxin-dependent peroxide	↑ (1.47)	NM_006793.2/NP_006784.1 A mitochondrial protein with antioxidant function it confers a	N	

Protein (Gene)	Avg. ratio	Protein function (taken from Gene Pubmed)	Relationship to Migration	Reference
reductase, mitochondrial (PRDX3)		protective role in cells through its peroxidase activity by reducing hydrogen peroxide, peroxynitrite, and organic hydroperoxides. Gene ID: 10935, updated on 23-Sep-2012		
Tubulin beta chain (TUBB)	↑ (1.23)	NM_178014.2/NP_821133.1 Part of the tubulin superfamily, beta-tubulin, with alpha-tubulin, make up the major components of microtubules Gene ID: 203068, updated on 9-Sep-2012	Upregulated in metastatic axillary lymph node breast cancer compared to primary tumours.	[615]
Ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL1)	↑ (4.19)	NM_004181.4/NP_004172.2 Belongs to the peptidase C12 family. This enzyme is a thiol protease that hydrolyzes a peptide bond at the C-terminal glycine of ubiquitin. Gene ID: 7345, updated on 15-Sep-2012	N	

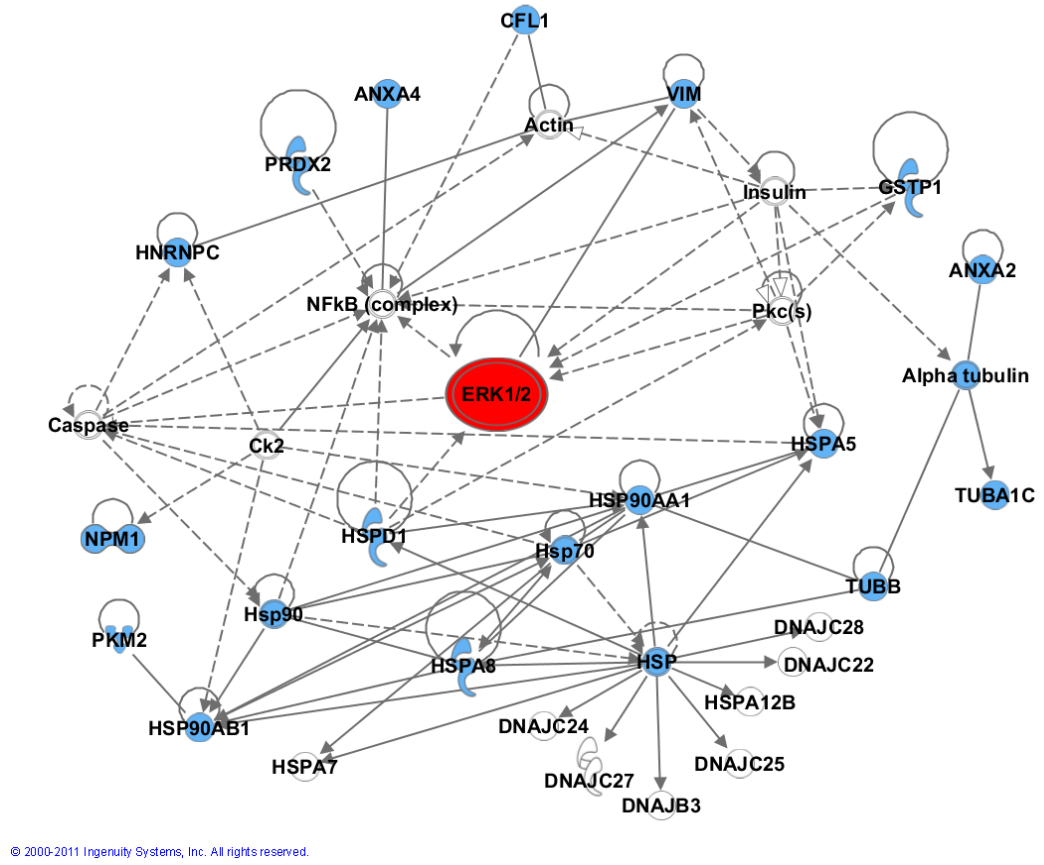

Figure 5.2 The top biological network identified using IPA analysis highlights ERK1/2 (shown in red) as a central hub relating to other proteins from the connectivity map. Nodes with blue background are the proteins differentially regulated by obestatin treatment in the SKOV3 ovarian cancer cell line. A line indicates interactions, with the arrow-head indicating the direction of the interaction (*i.e.* the protein that is being influenced). The absence of arrowheads refers to a binding interaction. A dotted line indicates an inferred or indirect interaction.

Table 5.4 Top two networks generated, from the identified proteins, by the Ingenuity Pathway Analysis software. Proteins that were differentially regulated by obestatin treatment in response obestatin treatment were entered into the IPA software for network generation and analysis. Proteins shown in **bold** are differentially expressed proteins which were identified in this study. The score is a number generated by the Ingenuity Pathway Analysis software to represent the likelihood that the proteins are grouped together functionally in the network, rather than being discovered together by chance, with a 99% confidence level. The higher the score the stronger the grouping, and scores ≥3 are considered to be significant. Focus molecules indicate the number of proteins from the list of proteins entered into the analysis that are represented in the network.

ID	Molecules in Network	Score	Focus Molecules	Top Functions of pathway
1	Actin, Alpha tubulin, **ANXA2, ANXA4,** Caspase, **CFL1,** Ck2, DNAJB3, DNAJC22, DNAJC25, DNAJC28, ERK1/2, **GSTP1, HNRNPC,** HSP, Hsp70, Hsp90, **HSP90AA1, HSP90AB1, HSPA5,** HSPA7, **HSPA8,** HSPA12B, **HSPD1,** Insulin, NFkB (complex), **NPM1,** Pkc(s), **PKM2, PRDX2, TUBA1C, TUBB, VIM**	42	16	Cancer, Gastrointestinal Disease, Post-Translational Modification
2	AGTR1, ANKRD12, **ARPC5L,** BAI3, CDKN2A, CLEC2A, **CPD,** Cytochrome c, **EEF1D, EIF4A1,** EIF4E, EMR3, **ENO1,** FFAR3, GIP2 (human), GPR21, GPR87, HS3ST1, IFNG, LGMN, MORC2, Myb, MYC, **PPA1, PRDX3, PSMA5,** RARRES1, Rpl9 (includes others), RT1-B, SCPEP1, TP53, tretinoin, **UCHL1,** VSIG4, ZNF385A	20	9	Cell Cycle, Cell Death, Cancer

known protein interactions (Fig. 5.2 and Table 5.4). Proteins were also associated with a network which consists of a number of molecules related to cell cycle control, cell death and cancer (Table 5.4).

5.3.2 Obestatin stimulates a reduction in ERK1/2 phosphorylation in ovarian cancer cell lines

In this study, the application of proteomic approaches was used to demonstrate that a number of proteins were differentially expressed in response to obestatin treatment in the SKOV3 ovarian cancer cell line. Further analysis using the IPA software identified the ERK1/2 MAPK pathway as a potentially important mediator of obestatin action, as it was associated with a number of proteins that were regulated by obestatin treatment. To determine if obestatin also stimulated the activation (phosphorylation) of the ERK1/2 signalling pathway Western blot analyses were performed using anti-active antibodies. Time-points with the most significant responses to obestatin were chosen after performing time course assays (for 0, 5, 15, 30, 45, or 60 min). Experiments were then performed to investigate ERK1/2 signalling in response to a range of concentrations of obestatin in the SKOV3 and OV-MZ-6 ovarian cancer cell lines (Fig. 5.3). Western blot analysis of a range of obestatin concentrations showed that ERK1/2 phosphorylation was significantly decreased with the 1000 nM obestatin treatment in both the SKOV3 (0.69 fold +/- 0.25) and OV-MZ-6 (0.40 fold +/- 0.18) cell lines and with the 100 nM treatment in the SKOV3 cell line (0.78 fold +/- 0.10).

To confirm this result using a highly quantitative assay, the effects of obestatin treatment on ERK1/2 signalling in the SKOV3 ovarian cancer cell line was performed across a range of time-points using a phospho-ERK1/2 ELISA kit (Sigma-Aldrich). Serum starved SKOV3 cells were treated with 0, 10 and 100 nM concentrations of obestatin for 5-30 min (Fig. 5.4). No statistically significant changes in phosphorylation were observed with obestatin treatment, compared to the untreated control.

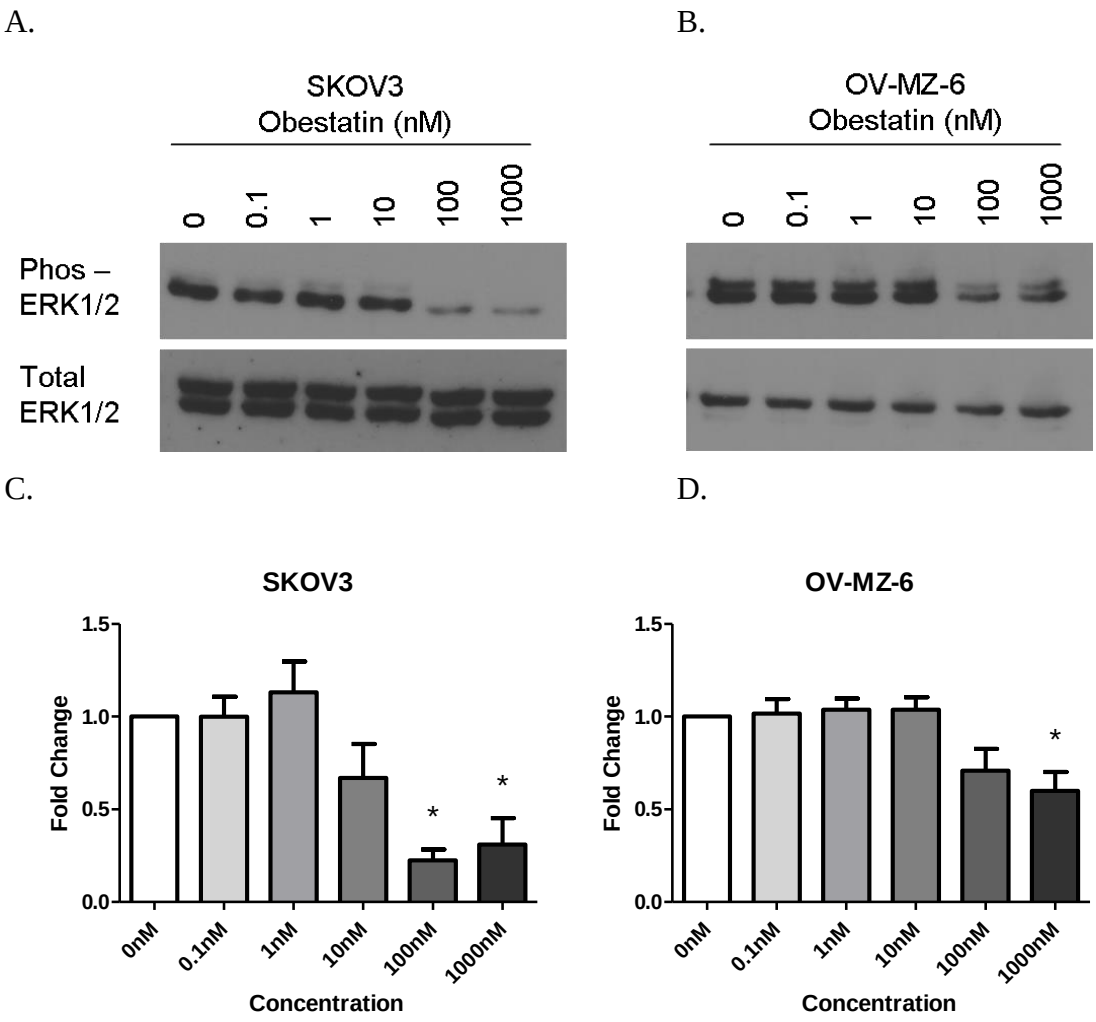

Figure 5.3 Western immunoblot analysis of ERK1/2 phosphorylation after obestatin treatment in the SKOV3 and OV-MZ-6 ovarian cancer cell lines. Serum-starved cells were treated with a range of obestatin concentrations (0, 0.1, 1, 10,100, and 1000 nM) for 15 min in the SKOV3 cell line and 30 mins in the OV-MZ-6 cell line (determined as the peak ERK1/2 signalling timepoints for these cell lines, see Chapter 2). Representative Western immunoblot of triplicate experiments showing cell lysates analysed using anti-phospho ERK1/2 and anti-Total ERK antibodies in the (A) SKOV3 and (B) OV-MZ-6 ovarian cancer cell lines. Densitometry was performed to quantify the changes in protein expression with different obestatin treatments in the (C) SKOV3 and (D) OV-MZ-6 cell lines. The densitometry data are an average of the triplicate experiment expressed as histograms (mean ±SEM) corrected total ERK1/2 in each protein extract. *P < 0.05 compared to 0 nM control.

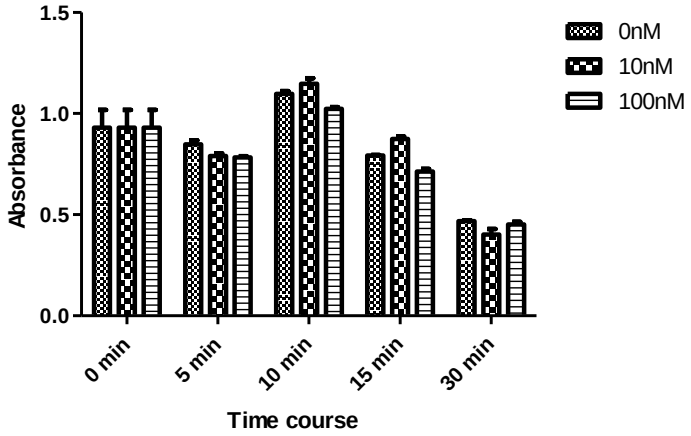

Figure 5.4 Investigation of ERK1/2 using Sigma Phospho-Erk1 ($pThr^{202}/pTyr^{204}$) + Erk2 ($pTyr^{185/187}$) and pan-Erk1/2 ELISA Kit in the SKOV3 ovarian cancer cell line. Serum-starved cells were treated with 0, 10 and 100 nM concentrations of obestatin for 0, 5, 10, 15 and 30 min. Graph represents a single experiment with phosphorylated ERK1/2 absorbance values normalised to pan ERK1/2 (mean ±SEM). Samples were assayed in duplicate (n=2) for each treatment at each timepoint.

5.4 Discussion

Using a proteomic approach in this study, we identified 26 proteins that were differentially regulated by obestatin compared to medium-only controls. The differentially expressed proteins were analysed using the Ingenuity Pathway Analysis (IPA) software to predict the pathways that are activated by obestatin. The two top networks associated with obestatin treatment that were identified were cancer progression and cell cycle regulation, providing further evidence that obestatin plays a role in cancer progression.

Using IPA, the ERK1/2 pathway was determined to be a key, central component in the mediation of these functions, and, therefore, this signalling pathway was further investigated in the ovarian cancer cell lines. Surprisingly, however, although treatment with obestatin was expected to activate ERK1/2 phosphorylation in the OV-MZ-6 or SKOV3 cell lines, it decreased phosphorylation at the time points tested. Taken together, these data suggest that obestatin, like ghrelin, could play a role in ovarian cancer, stimulating cell migration, but not via the ERK1/2 signalling

pathway.

2D-DIGE proteomic analysis of normal (hOSE 17.1) and ovarian cancer (SKOV3) cells treated with (+/-) obestatin revealed that a number of proteins were differentially expressed. On the basis of the known or putative functions of these proteins, they can be grouped into a number of broad functional categories; including roles in the cytoskeleton, cellular motility and plasticity (vimentin, tubulin alpha-1C chain, tubulin beta chain, ARPC5L, cofilin-1, annexins), protein folding, modification and degradation (glucose regulated protein 78, proteasome subunit alpha type-5, heat shock proteins, ubiquitin carboxyl-terminal hydrolase isozyme L1, carboxypeptidase D), metabolism and enzymatic capabilities (glutathione S-transferase P, alpha-enolase, pyruvate kinase isozyme M1/M2), transcription/translation factors (EEF1D, EIF4A1, heterogeneous nuclear ribonucleoproteins), regulation of apoptosis (nucleophosmin, heat shock proteins) and antioxidant proteins (peroxiredoxin-2, thioredoxin-dependent peroxide reductase).

Some of the proteins identified in this study have previously been associated with cell migration in other studies (Tables 5.2 and 5.3). Additionally, a number of the proteins that were differentially expressed in this study in response to obestatin treatment have also been previously reported to be associated with increased cell migration, or related functions. Increases in annexin A4 [607], CFL1 [608-611] and GSTP1 [612] expression, or inhibition of hnRNPK [597], HSPD1/HSP60 [588] and HSPA5 [586, 588, 589] have been shown to stimulate cell migration. Increased expression of annexin 4 in renal clear cell carcinoma cells was shown to increase cell migration [607] and annexin 4 may also play a role in increased cell migration in SKOV3 cells. A similar link between annexin A4 and migration was found in glioma cells, where it was shown that microRNA-7 decreased annexin 4 expression and decreased migration in the glioma cell lines [606]. In neck cancer cell lines, the combined knockdown/inhibition of HSPD1 and HSPA5 was found to considerably enhance cell migration [588]. Knockdown of these proteins in ovarian cancer cell lines, followed treatment with obestatin, would help to determine the role of these proteins in obestatin-stimulated cell migration.

For some of these proteins there has been conflicting data regarding their role in cell migration, however, suggesting that there may be tissue specific responses. In head and neck cancer, knockdown of the gene for Heat shock protein 60, HSPD1 was correlated with increased migration [588]. Similarly, in our study obestatin led to a decrease in Hsp60 protein expression and this coincided with increased cell migration. In purified human T cells [591] and the AGS gastric cancer cell line [592], however, Hsp60 treatment was linked to enhanced migration. Not all of the changes observed in our study are consistent with an increase in cell migration with obestatin treatment, and obestatin may have additional functions in ovarian cancer cell lines. Although not all of the differentially regulated proteins were associated with cell migration, some proteins, including EIF4A [584] and TUBB [615] have been linked to metastasis.

A number of proteins, that have been associated with increased cell migration, were regulated by obestatin, but the response did not support their role in obestatin-mediated migration. It is likely that obestatin has other cellular effects in addition to the stimulation of cell migration. An upregulation of alpha-enolase [577, 578], annexin A2 [579], Hsp90 alpha and Hsp90 beta [594, 595], nucleophosmin [598-601], and vimentin [586, 587, 602-605] is usually associated with an increase in cell migration, however, their expression in response to obestatin was decreased in the current study (Table 5.3). Conversely, periredoxin-2 was elevated with obestatin treatment (Table 5.4), however, in vascular remodelling a deficiency of PRDX2 indirectly enhanced cell migration [614]. Aloe-emodin treatment of the HepG2 cell line enhanced oxidation of PRDX2 and was associated with decreased migration [613]. The majority of these studies, however, observed these proteins during interactions with other proteins and/or drugs and their effect and response may differ under different circumstances.

An increase in vimentin expression is frequently associated with increased migration [586, 587, 602-605], but protein expression was reduced in the SKOV3 and hOSE 17.1 cell lines in response to obestatin treatment. Upregulation of vimentin expression coincided with the downregulation GRP78 in hepatocellular carcinoma cells and is associated with increased cell migration [586, 587], whereas in the current work, obestatin treatment downregulated both proteins. In a study on aortic

valve disease the secretion of cytokines by invading inflammatory cells induced myofibroblastic activation, characterised by decreased vimentin expression and increased migration [616].

Although obestatin stimulates cell migration in the SKOV3 ovarian cancer cell line, as indicated not all of the changes seen in protein expression correlated with expected changes. In this study, cells were treated with obestatin for 3 days (and this was consistent with the pre-treatment and treatment for migration assays) in flasks (80-90% confluent upon harvest) and migrating cells were not harvested from Transwell migration assays. The collection of cells in an assay that allows cell migration in response to obestatin would select for proteins related to this process. To investigate this, the 2D-DIGE experiment and analysis could be repeated with obestatin treated cells in Transwell assays and gene expression could also be determined using oligonucleotide microarrays.

With obestatin treatment, significant increases in cell invasion were also observed in the OV-MZ-6 ovarian cancer cell line and the hOSE 17.1 normal ovarian cell line, but not in the SKOV3 cell line (see Chapter 4). While a number of molecules associated with cell invasion were differentially regulated by obestatin, including annexin A2 [579, 617], HSP60 [588], GRP78 [588], α-enolase [577] and nucleophosmin [599], the direction of change (up or downregulation) did not correspond with previously described changes. In the current study, obestatin treatment caused a decrease in annexin A2, HSP60, GPR78, α-enolase and nucleophosmin. Conversely, accumulation, or activation, of nucleophosmin has been linked to a more invasive phenotype [599-601]. The combined downregulation of HSP60 and GRP78 significantly reduced invasiveness of head and neck cancer cell lines [588]. Similarly, inhibition of α-enolase [577] decreased invasive potential of the MDA-MB-231 breast cancer cell line. Annexin A2 is normally up regulated on the cell surface in cancer cells, influencing cancer related processes including angiogenesis, proliferation, apoptosis, adhesion, cell migration and also increasing cell invasion [617]. In ovarian cancer, annexin A2 is upregulated in the conditioned medium from cultured ovarian cancer cells. In the current study proteins were measured from cell lysates, and this could explain the observed difference in expression for this protein [617]. As discussed earlier, the difference in expression of

these proteins observed from the results of the 2D-DIGE, compared to other studies, may be the result of the cells not being collected under migrating conditions. Subsequently, this may change their response to obestatin if they were migrating upon collection.

Additionally, the cancer related proteins, α-enolase and hnRNP K, were downregulated by obestatin in this study (Table 5.3). Alpha-enolase is upregulated in cancer, supporting anaerobic proliferation and invasion of the tumour [577]. hnRNPK, a multifunctional signalling protein, has been shown to be crucial for metastasis in HT1080 (human fibrosarcoma) and HEK293T (human embryonic kidney carcinoma) cancer cell lines [596]. The expression of these proteins may change if collected from cells in a migration assay.

It is important to consider that the differentially expressed proteins were identified by 2D-DIGE, and therefore, their change in expression is not necessarily due to a change in the level of protein expression. Changes in apparent abundance of a protein in response to a treatment may result from a shift in the position of a protein on a gel due to altered post-translational modifications, causing changes in pI or the size of the protein. In a two-dimensional gel electrophoresis (2DGE) analysis of oxidative stress in cardiac myocytes, for example, changes in the abundance of peroxiredoxins and two small heat shock protein (Hsp) family members were observed, however, this was not due to a change in protein level [618], but due to altered protein modification. The shift in abundance of peroxiredoxins is hypothesized to result from the over-oxidation of active site Cys residues. The Hsp may have become phosphorylated, causing a shift to lower pI values [618]. The observed upregulation of cofilin-1 after obestatin treatment may also indicate a change in phosphorylation status. Phosphorylation of cofilin-1 inactivates it and would also change its pI value. An increase in cofilin-1, in addition to a reduction in its phosphorylated form, was linked to the stimulation of cell migration in human colon adenocarcinoma cell lines compared to more motile adenocarcinoma sublines (EB3, 3LNLN, 5W) and cell lines derived from the parental LS180 adenocarcinoma cell line and the fibroblastic normal rat kidney (NRK) cell line [609]. Increased migration was also correlated with decreased cofilin-1 phosphorylation in nasopharyngeal carcinoma cell lines in response to siRNA knockdown of p57kip2, a

CDK inhibitor [610]. Similarly, increases in the phosphorylated form of this protein inhibited cell migration in breast cancer cell lines [611]. It is possible that obestatin could have effects by influencing protein modifications.

2D-DIGE analysis without prior fractionation of the sample may lead to under-representation of low abundance and small proteins. Further fractionation of the sample prior to 2D separation can increase the protein coverage of the method and allow the identification of these proteins and the application of other methods can complement this method [619]. Liquid chromatographic separation, coupled with mass spectrometry and labelling of proteins (or label-free methods), can lead to the identification of a different range of differentially expressed proteins and complementary coverage of the proteome and further studies could employ these methods.

The IPA of the 26 proteins identified the ERK1/2 pathways as major mediating pathway for the obestatin regulated proteins. Western blot analysis in the ovarian cancer cell lines showed a significant decrease in ERK1/2 phosphorylation with 100 nM and 1000 nM obestatin treatments, indicating that treatment downregulated this signalling pathway. This contrasts the results of the 2D-DIGE experiment which showed the most significant changes were induced by 10 nM obestain treatment, while significant downregulation of ERK1/2 phosphorylation occurred with 100 and 1000-nM treatments. This discrepancy may be attributed to an accumulative effect in the 2D-DIGE experiment, as these cells received 3 treatments of obestatin, as opposed to the signalling study where the cell received a single treatment. Additionally, it should be noted that expression of the ERK1/2 protein itself was not demonstrated using 2D DIGE, although it was a central pathway identified using pathway analysis. Quantitative assays using ERK1/2 ELISAs did not demonstrate a significant change in phosphoyrlation in response to obestatin treatment at a range of time points in the SKOV3 cell line. Although these results appear to contradict the software analysis it should be noted that the IPA analysis draws on information from a database of all known protein interactions from the literature, in all tissue types. The responses we observed may be specific to the ovary. In the previous chapter, it was demonstrated that obestatin significantly increased cell migration in both the SKOV3 and OV-MZ-6 ovarian cancer cell lines, but not the hOSE 17.1 normal

ovarian cell line (Chapter 4.3.3). These results suggest that it is unlikely that obestatin-stimulated migration in the ovarian cancer cell lines is mediated by the ERK1/2 pathway. Obestatin signalling pathways have not been widely explored [7]. ERK1/2 has previously been shown to be associated with obestatin signalling in cell proliferation and the anti-apoptotic effects of obestatin [10-12, 45, 462, 465, 466], but there have been no studies demonstrating that obestatin stimulates cell migration.

ERK1/2 phosphorylation was determined 15 mins after treatment in the SKOV3 cell line and 30 mins after treatment in the OV-MZ-6 cell line, as the overall peak signalling response for ERK1/2 was seen at these timepoints. This is consistent with another study in porcine ovarian co-culture cells that observed maximum stimulation of phospho-ERK1/2 levels after a 15 min incubation [509]. Further studies are required to investigate of ERK1/2 signalling at a range of timepoints, however, would confirm the lack stimulation is not due to the timepoint chosen. Using a phospho-ERK1/2 ELISA kit (Sigma), no statistically significant change in phosphorylation was observed with treatment. This study also suggests that ERK1/2 signalling is not associated with obestatin-stimulated cell migration.

Through IPA, the PKC signalling pathway and the NF-κB signalling complex were associated with obestatin treatment. These two pathways have been previously shown to be involved in ghrelin-stimulated migration [16, 55] and should be investigated as pathways that may regulate obestatin-stimulated migration in ovarian cancer cell lines.

This is the first report showing that the ghrelin-gene derived peptide, obestatin, differentially regulates a range of proteins related to cancer and cell functions, including cell migration, providing evidence that obestatin has a role in ovarian cancer.

CHAPTER 6

General Discussion

6.1 Discussion

There is an expanding body of research providing evidence for a role of the ghrelin axis in cancer development and progression [15], and this study has provided evidence that it has a role in ovarian cancer. This study has demonstrated for the first time that both ghrelin and obestatin promote cell migration in the SKOV3 and OV-MZ-6 serous ovarian cancer cell lines, but not in the hOSE 17.1 normal ovarian epithelial cell line, and these peptides may play a role in ovarian cancer progression. Ghrelin treatment also decreased SKOV3 attachment to collagen IV. In both the SKOV3 and OV-MZ-6 ovarian cancer cell lines, obestatin treatment led to a decrease in ERK1/2 phosphorylation. Obestatin also stimulated invasion in the OV-MZ-6 ovarian cancer and hOSE 17.1 normal ovarian cell lines, however, no change in invasion was observed in the SKOV3 ovarian cancer cell line. Interestingly, neither ghrelin or obestatin had any effect on proliferation in any of the cell lines studied, although they have been shown to influence cell proliferation in a number of other types [7, 15].

It is well established that ghrelin is an orexigen and a growth hormone secretagogue, however, this multi-functional peptide also has roles in cardiac function, immunity, growth, appetite, sleep and cellular functions including proliferation, migration and apoptosis [4, 15, 381]. The peptide, obestatin, is also derived from the ghrelin gene and the ghrelin preprohormone [6]. Despite initial controversy, a number of studies have demonstrated that obestatin, like ghrelin, has a diverse range of functions and some roles may be similar to those of ghrelin [7]. Components of the ghrelin axis are expressed in a wide range of cancer types, and in some cases, expression is altered in cancer (see Chapter 1) [15]. Ghrelin and obestatin have been shown to influence a number of cellular functions associated with the hallmarks of cancer [7, 15].

In this thesis, the expression and function of ghrelin and the preproghrelin derived hormone, obestatin, were investigated in ovarian cancer. There is some evidence that ghrelin and obestatin levels may be elevated in ovarian cancer. In a small, independent study of patients undergoing surgery for benign ovarian tumours (22 patients), or ovarian cancer (31 patients), plasma concentrations of both acylated ghrelin and obestatin were elevated, compared to 32 control women. This suggested that these peptides could play a role in ovarian cancer, and obestatin could be a

useful biomarker for ovarian cancer [114]. There were, however, a number of limitations to this study, including small sample size and the measurement of acylated ghrelin may have been unreliable due to the pre-treatment of the plasma samples [114]. In addition, acyl ghrelin levels were not correlated with BMI in this study. An immunohistochemical study of serous ovarian tumours observed increased expression of ghrelin, but not obestatin, in malignant samples compared to benign tissues [380]. We investigated the expression of ghrelin, GHSR1a and obestatin mRNA in ovarian cancers (including serous, papillary serous, endometrioid, clear cell and mucinous adenocarcinomas) of a range of stages (from I to IV) and in ovarian cancer cell lines (Chapter 3.3.1 and 4.3.1). Although expression of ghrelin and obestatin transcripts was demonstrated in normal and tumour tissue, their expression was not correlated with cancer grade, or consistently elevated in all samples compared to the normal tissue. Our data suggests that obestatin and ghrelin expression may be upregulated in a subset of ovarian cancers, however.

As most ovarian cancers are derived from the single layer of ovarian surface epithelium (which is often detached during dissection of the ovary), and the normal samples in this study are derived from whole ovary, which is a heterogenous organ, it was not possible to compare expression of normal ovarian epithelium and cancer using RT-PCR in this study, however. Using immunohistochemistry for obestatin we have demonstrated that obestatin is expressed at very low or absent levels in ovarian surface epithelium, and expression is higher in ovarian cancers and benign tumours. A recent study also showed no change in obestatin expression, but an increase in ghrelin expression in malignant serous ovarian tumours tissues compared to benign tissues [380]. As this study only investigated one type of ovarian cancer, serous, further immunohistochemical studies of ghrelin expression in other types of ovarian cancer and benign tissue are required.

Interestingly, the cell lines studied expressed the GHSR1b (the truncated ghrelin receptor) isoform, but not the ghrelin receptor, GSHR1a. This is consistent with our previous studies showing that high grade serous cystadenocarcinomas did not express GHSR1a [45]. Although we have demonstrated that GHSR1a is not expressed at the mRNA level in a panel of ovarian tumours, we have previously demonstrated GHSR1a expression in ovarian cancer using an immunohistochemical

approach [45] and these results are difficult to reconcile. It is surprising that the GHSR1a protein was demonstrated in ovarian cancer [45], but the mRNA is not expressed. The mRNA isoforms expressed by ovarian cancer may be different to the wildtype receptor isoform and could not be detected using our RT-PCR assays, which we have used to demonstrate GHSR1a expression in prostate [39], breast [17] and endometrial cancer [49]. Conversely, the antibodies used in immunohistochemical studies were generated against the C-terminal region of the GHSR1a and it might cross react with undescribed variants of the receptor, or it may lack specificity and bind to related GPCRs. Further protein analysis, using more sensitive and specific methods, such as tandem mass spectrometry, are required to investigate these results.

As the ovarian cell lines in this study do not appear to express the GHSR1a, it is likely that the effects of ghrelin are mediated through the alternative ghrelin receptor. Given the identity of this receptor is unknown, we are unable to investigate this further by targeting the alternative receptor specifically. It would be useful to determine if these cell lines showed the same response to desacyl ghrelin treatment, as it acts through the alternative ghrelin receptor. The use of GHSR1a antagonists to ensure inhibition of any residual GHSR1a action, may also prove difficult, as it is possible that the alternative receptor may have similar binding sites to GHSR1a. Indeed, the GHSR1a inverse agonist, D-Lys3-GHRP-6, has been shown to inhibit the action of desacyl ghrelin [549]. By reintroducing the GHSR1a receptor to these cell lines using expression constructs, we can investigate whether any of the results seen may be altered due to the overexpression of GHSR1a. Similarly, the identity of the obestatin receptor is currently unknown, although it appears to be a G protein coupled receptor. Although the identity of the receptor is unknown, the signalling pathways involved in obestatin function in ovarian cancer could be investigated further.

There is evidence that ghrelin and obestatin may play a role in a number of processes related to cancer progression [7, 15, 381]. The progression and development of cancer is characterised by a number of predictable steps and processes, defined as the hallmarks of cancer [392, 393]. These hallmarks of cancer include self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of

programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and increased tissue invasion and metastasis [392, 393]. Few studies have investigated the role of obestatin in cancer. As obestatin levels may be elevated in the circulation of patients with ovarian tumours [114], and it is expressed in ovarian cancer cells [380, 465, 620], (Chapter 4), we hypothesise that it may play an endocrine or autocrine/paracrine role in ovarian cancer.

The most significant finding of this study was that both ghrelin and obestatin increased cell migration by approximately 2 fold in the SKOV3 and OV-MZ-6 ovarian cancer cell lines. This is the first demonstration that the peptide hormone, obestatin, stimulates cell migration in any cell line, and this provides a novel avenue for further research. The response to both acylated ghrelin and obestatin treatment was investigated in two ovarian cancer cell lines and a normal cell line in this study, and indicated that the response may be cancer specific, however, further studies in additional normal and cancer cell lines are required. In this study, ovarian cancer cell lines were derived from serous ovarian adenocarcinomas cells that had metastasised to the ascites, rather than from the primary tumour site, and thus, they are likely to have been an actively migrating phenotype. Cell lines representing a number of other ovarian cancer subtypes could also be tested. *In vivo* xenograft metastasis assays, including the injection of cells into the peritoneal cavity of immunocompromised mice, could provide further insights into the ability of ovarian cancer cells to migrate and metastasise in response to acylated ghrelin and obestatin.

Although ghrelin has been demonstrated to increase cell migration in other cancer cell lines [48, 54], this is the first demonstration that exogenous ghrelin treatment increases cell migration in ovarian cancer cell lines. In a previous study using Boyden chambers (BD Bioscience, Bedford, MA), increased migration and invasion (Matrigel coated) was seen in poorly differentiated PANC1 and MIAPaCa2, and well-differentiated BxPC3 and Capan2 human pancreatic cancer cell lines with ghrelin treatment [54]. Treatment with the GHSR inverse agonist, D-Lys-3-GHRP-6, inhibited ghrelin-stimulated cell migration [54]. Inhibition of migration using D-Lys-3-GHRP-6 suggests that ghrelin-stimulated cell migration may be mediated by GHSR1a, and both GHSR1a and GHSR1b were expressed in these pancreatic cancer cell lines [48, 54]. It is possible, however, that this inverse agonist may also bind the

alternative ghrelin receptor as it has been shown to inhibit the response to desacyl ghrelin [210, 549], which does not act through the GHSR1a receptor. Treatment with an antibody against endogenous ghrelin in the SW-48 and ROK colon cancer cell lines also inhibited cell migration [48]. Similar studies could be performed in ovarian cell lines to determine if the GHSR1a and endogenous (autocrine/paracrine) ghrelin play a role in ovarian cancer cell migration, although our studies indicate that this receptor might not be expressed in ovarian cancer. We hypothesise that in ovarian cancer, ghrelin stimulates cell migration through the alternative ghrelin receptor, the identity of which is currently unknown.

This is the first study to indicate that obestatin may play a role in cell migration. In this study, we investigated the effect of obestatin treatment on protein expression using 2D-DIGE (two dimensional-difference in gel electrophoresis) and mass spectroscopy (Chapter 5.3.1) to provide insight into the mechanism of action of obestatin. This study revealed that 26 proteins were differentially expressed by the obestatin-treated SKOV3 ovarian cancer cell line, compared to the untreated controls. Proteins that were regulated by obestatin treatment were analysed using Ingenuity Pathway Analysis. The top two networks identified suggested that the proteins identified from the 2D-DIGE experiment were likely to be involved in cellular processes including post-translational modification, the cell cycle and cell death. The differentially expressed proteins belonged to a number of functional groups. Some were associated with the cytoskeleton, cellular motility and plasticity and included: vimentin, tubulin alpha-1C chain, tubulin beta chain, ARPC5L, cofilin-1 and the annexins. Other proteins were identified as having a role in protein folding, modification and degradation (including GRP78, proteasome subunit alpha type-5, heat shock proteins, ubiquitin carboxyl-terminal hydrolase isozyme L1, carboxypeptidase D), or as having metabolic and enzymatic capabilities (including glutathione S-transferase P, alpha-enolase, pyruvate kinase isozyme M1/M2). As expected, given the fact that obestatin stimulated cell migration in our *in vitro* studies, a number of these proteins were identified as being involved in cell migration, processes related to migration or were other cancer related proteins. These findings warrant further investigation by Western blot analysis and inhibitor assays to confirm their involvement and differential expression. Further validation in the SKOV3 and other ovarian cancer cell lines will be useful in confirming these results,

and knockdown studies could be used to investigate the role of these molecules in obestatin-stimulated cell migration. Obestatin may have other effects on cell physiology that have not previously been identified, and not all of the differentially expressed proteins identified in this study would be directly related to its role in cell migration, however.

Ingenuity pathway analysis indicated that obestatin may regulate cell death in ovarian cancer cell lines. Obestatin has been shown to have anti-apoptotic effects in cardiomyocytes [462] and HIT-T15 and INS-1E beta cell pancreatic cell lines [12]. Obestatin has been associated with increased apoptosis in porcine ovarian granulosa cells, which showed increased expression of apoptotic markers Bax, p53 and Caspase 3 [465]. Interestingly, none of these markers were identified in the current study, however, cell death was a process identified by the Ingenuity Pathway Analysis. Obestatin may, therefore, play a role in regulating apoptosis in ovarian cancer cell lines, and this should be investigated using functional assays.

As obestatin has not previously been shown to stimulate cell migration, the signalling pathways involved were investigated in this study. The ERK1/2 signalling protein was identified as a key molecule in response to obestatin treatment in this study using 2D-DIGE and Ingenuity Pathway Analysis (Chapter 5.3.1) and, therefore, the effect of obestatin on ERK1/2 signalling was investigated. Western immunoblot analysis was performed in order to investigate the effects of obestatin on ERK1/2 phosphorylation. While an increase in phosphorylation would be expected if obestatin signals through this pathway, there was no increase in ERK1/2 phosphorylation with obestatin treatment, and a decrease in phosphorylation was observed at higher concentrations. This suggests that ERK1/2 is not a signalling pathway stimulated by obestatin in ovarian cancer and it is unlikely to be playing a part in obestatin-stimulated cell migration.

Ghrelin treatment had a similar effect on ERK1/2 signalling, showing no change in phosphorylation with treatment and decreased phosphorylation in the SKOV3 ovarian cancer cell line. The ERK1/2 pathway is a recognised as an important ghrelin signalling pathway, and is stimulated by ghrelin in porcine ovarian follicular [509] and granulosa cells [395], chicken ovarian granulosa cells [508, 621] and the SKOV3

ovarian cancer cell line [524], although the effects of ghrelin on ERK1/2 phosphorylation in human ovarian cancer have not been previously investigated. The results of this study, however, suggest that that ERK1/2 does not play a role in the response of ovarian cancer cell lines to ghrelin. In endothelial cells, however, ghrelin-mediated cell migration coincided with increased phosphorylation of ERK1/2 [171, 172, 174].

As it appears to be unlikely that ERK1/2 is involved in signalling and cell migration in response to ghrelin and obestatin, other pathways need to be investigated. There have been a few studies investigating the signalling pathways that are activated by ghrelin which lead to cell migration. Ghrelin increases migration in a range of endothelial cells by stimulating increased calcium mobilisation [190]. Migration in these cells could be attenuated by cAMP and protein kinase A (PKA) inhibitors [190]. A study of astrocytoma cell lines, U-118, U-87, CCFSTTG1 and SW1008, also showed that calcium mobilisation increased, and protein kinase C (PKC) was stimulated in response to ghrelin treatment [16]. Ghrelin-stimulated migration in rat C6 and human U251 glioma cell lines was mediated through the calmodulin-dependent protein kinase II (CaMKII), AMP-activated protein kinase (AMPK), and nuclear factor-kappa B (NF-κB) pathways [55], and these pathways could also be involved in ghrelin stimulated cell migration in ovarian cell lines. Some ghrelin signalling molecules and pathways, including PKA [12], PKC [10, 11, 462, 466], ERK1/2 [10-12, 462, 465, 466] and AMPK [464], are also activated by obestatin (as discussed in Chapter 1.4.3). Interestingly, the PKC pathway and the NF-κB complex were also associated with the network of proteins identified from the 2D-DIGE experiment, generated in the Ingenuity Pathway Analysis (discussed in Chapter 5.3.1). This 2D-DIGE method did not investigate the activation (phosphorylation) of signalling molecules, however. These signalling pathways should be investigated for their potential involvement in ghrelin- and obestatin-stimulated cell migration. This could be achieved using Western blot analyses or phosphorylated protein arrays to demonstrate activation (phosphorylation) in response to treatment with ghrelin and obestatin. It would be useful to perform Transwell migration assays in the presence of signalling pathway inhibitors to determine their role in migration. It is likely that a number of signalling pathways mediate the response to obestatin and ghrelin.

The role of ghrelin in cell invasion, a hallmark of cancer that is essential for metastasis, has been the subject of little study, and there have been no studies into the role of obestatin in invasion. Ghrelin increases the invasiveness of the poorly differentiated PANC1 and MIAPaCa2, the well-differentiated BxPC3 and Capan2 pancreatic adenocarcinoma cell lines [54] and the SW-48 and ROK colorectal cancer cell lines [48]. Ghrelin treatment caused no change in cell invasion in the normal ovarian and ovarian cancer cell lines in this study. Obestatin treatment did, however, cause a significant increase in invasion in the OV-MZ-6 ovarian cancer cell line and the hOSE 17.1 normal ovarian cell line. This suggests that obestatin may play a role in normal ovarian epithelial cell invasion, and a study in a wider range of cell lines is required

Cell attachment is also a key process in cancer progression. Epithelial ovarian cancer spreads primarily by the implantation of spheroids of tumour cells onto the mesothelium that lines the peritoneal cavity [527, 528]. The ovarian cancer cells attach to mesothelial cells by binding to a number of molecules, including extracellular matrix (ECM) ligands such as fibronectin which are expressed by the mesothelial cells themselves [551]. Mesothelial cells secrete an array of ECM molecules, including fibronectin, vitronectin and collagen I, expressing them on their cell surface and secreting them into the peritoneal cavity [552-554]. Cancer cells then gain access to the submesothelial extracellular matrix, allowing the cells to attach with a high affinity [551]. The role of ghrelin and obestatin in cancer cell attachment has not been studied. Obestatin had no effect on attachment to any of the ECM molecules tested in this study in any of the cell lines. Ghrelin also had no effect on cell attachment to the ECM molecules, fibronectin, vitronectin, collagen I, or collagen IV in the hOSE17.1 and OV-MZ-6 ovarian cell lines. In the SKOV3 ovarian cancer cell line, there was a significant decrease in attachment to collagen IV with 10 nM ghrelin treatment only. Although collagen IV is not normally expressed by mesothelial cells, it is expressed by the basement membrane and this could affect attachment to the submesothelial layer. As this result was only observed in one cell line and at one ghrelin concentration this finding might not be physiologically relevant and and further investigation may be warranted in other ovarian cancer cell lines of different cell types.

It has previously been demonstrated that ghrelin and obestatin play a role in cell proliferation [381]. Ghrelin influences cell proliferation in a large number of normal and cancer cell types [15]. We have previously shown that exogenous ghrelin treatment increases proliferation in breast [17], prostate [39, 40] and endometrial [49] cancer cell lines and ghrelin also increases cell proliferation in a number of cancer cell lines, colon [48], liver [25], pancreas [54, 56], pituitary [57] and thyroid [56]. In the ovary, studies of porcine ovarian follicular cells have demonstrated that ghrelin treatment increases cell proliferation [395, 509]. Obestatin, has also been shown to play a role, increasing cell proliferation in some cell lines, while having no effect in others [7]. In this thesis, however, neither peptide had an effect on proliferation in ovarian cancer cells or in the normal hOSE 17.1 cell line (Chapter 3.3.4 and 4.3.3). Although ghrelin has been shown to stimulate cell proliferation in a large number of cell lines, it has also been shown to have no effect on cell proliferation in a number of cell types, including the MCF-10A normal-derived breast cell line, the MCF7 breast cancer cell line [17], the KATO-III gastric cancer cell line [11], the SupT1 leukemic cell line [51] and the CALU-1 lung carcinoma cell line [27].

Initial studies suggest that obestatin may have similar functions to ghrelin, and it also increases cell proliferation in the KATO-III gastric cancer cell line [11] and prostate cancer cell lines (personal communication Dr Laura De Amorim, Ghrelin Research Group, QUT). Obestatin treatment had no effect on ovarian cancer cell proliferation in this study and obestatin also had no effect on cell proliferation in other studies in the BON-1 pancreatic neuroendocrine tumour cell line [56], the AGS gastric cancer cell line [466], or the GC rat somatotroph tumour cell line [466]. Ghrelin and obestatin may, therefore, increases cell migration, but has no influence on cell proliferation in the ovarian cancer cell lines in this study.

This study has focused on the role of ghrelin and obestatin in serous ovarian cancer cell lines compared to a normal ovarian surface epithelial cell line (hOSE 17.1). Serous carcinomas represent 75%-80% of ovarian cancer cases diagnosed [61]. Although other epithelial ovarian cancer subtypes are less common, it would be useful to perform these assays using cell lines representing other types of epithelial ovarian cancers and additional normal epithelial-derived ovarian cell lines. These studies will confirm whether the ghrelin axis may play a role in other ovarian cancer

types.

In conclusion, this thesis has demonstrated for the first time that ghrelin and obestatin may play a role in ovarian cancer progression. Treatment with the hormones, ghrelin or obestatin, stimulates a significant increase in cell migration, a key process related to metastasis, in human ovarian cancer cell lines. Obestatin increased cell invasion in the hOSE 17.1 cell line, which may suggest that it plays a role in the normal ovary and in the OV-MZ-6 cancer ce3ll line which may indicate that it promotes the invasion in cancer [392, 393]. Ovarian cancer is the leading cause of death among gynaecological cancers [58]. This is largely because two thirds of ovarian cancer cases are not diagnosed until the late stages of cancer development (either stage III or IV), after metastases have developed, resulting in limited treatment choices and a poor prognosis for the patient [61]. Our studies, indicating that ghrelin and obestatin play a role in cell migration, suggest that the ghrelin axis could be a useful future target for ovarian cancer treatment.

CHAPTER 7

References

7.1 References

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